

Chemoenzymatic Synthesis of Phosphorylated Glycoproteins

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Dedication

I would like to dedicate this thesis to my beloved parents

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Abstract

Phosphorylation of the glycan portion of glycoproteins is an important posttranslational modification. In particular, the presence of mannose-6-phosphate (M6P) residues on high mannose glycans of lysosomal enzymes is important for the transfer of these enzymes to the lysosomes. The synthesis of different types of *N*-glycan structures containing M6P residues have been reported by various groups. This thesis work concerns the chemoenzymatic synthesis of phosphorylated glycoproteins, wherein M6P containing *N*-glycans were synthesised chemically and then enzymatically coupled to give homogeneous glycoproteins.

Endo- β -N-acetylglucosaminidases (ENGases), have been employed by various research groups for the synthesis of variety of homogeneous glycopeptides and proteins by using oxazolines as activated donors. This thesis reports the synthesis of bis-phosphorylated tetrasaccharide and hexasaccharide oxazoline donors, and the use of ENGases to catalyse their transfer to peptides and proteins.

Chapter 1 provides an overview of the current literature regarding the synthesis of glycoproteins, with particular attention paid to the use of glycoprotein remodelling. A detailed description about lysosomal storage diseases and the commercially available enzyme replacement therapy (ERT) is included. The objectives of the project are outlined.

Chapter 2 and 3 details the synthesis of a bisphosphorylated tetrasaccharide and hexasaccharide oxazoline donors.

Chapter 4 reports the results of ENGase-catalysed glycosylation reactions using the phosphorylated tetrasaccharide and hexasaccharide oxazoline donors. Glycosylation of a model glycosyl amino acid acceptor was carried out with these donors using Endo A, Endo M and the commercially available Endo M mutant N175Q as the catalyst. Glycosylation of RNase B which had been trimmed back to contain only a GlcNAc at the single glycosylation (GlcNAc-RNase B) site using Endo A as catalyst with the oxazoline donors is reported. The binding of phosphorylated tetrasaccharide- RNaseB to the cation-independent mannose-6-phosphate receptor (CI-MPR) is demonstrated by the use of a fluorescent antibody assay. Finally, an investigation into glycoprotein remodeling using partially de-glycosylated Fabrazyme is also reported.

Chapter 5 contains the experimental details for compounds synthesised and procedures described in Chapters 2 to 4.

Abbreviations

The following abbreviations have been used in this thesis:

General:

Å	Ångstrom
Ac	Acetyl
ADP	Adenosine diphosphate
All	Allyl
Ar	Aromatic
ASGPR	Asialoglycoprotein receptor
AV	Adenovirus
aq	Aqueous
at	Apparent triplet
BF ₃ ·OEt ₂	Boron trifluoride diethyl etherate
Bn	Benzyl
br.	Broad
BSA	Bovine serum albumin
Bu	Butyl
Bz	Benzoyl
°C	Degrees Celsius
<i>c</i>	Concentration
Calcd.	Calculated
CAN	Ceric ammonium nitrate
Cbz (Z)	Benzyloxycarbonyl
CD-MPR	Cation-dependent mannose phosphate receptor
CHO	Chinese hamster ovary
CI-MPR	Cation-independent mannose phosphate receptor
cm	Centimetre
COSY	Correlation spectroscopy

CSA	Camphor sulfonic acid
Δ	Reflux
δ	Chemical shift (ppm)
d	Day (time)
d	Doublet (NMR)
Da	Dalton
DBU	1,8-Diazabicyclo[5.4.0]-7-undecene
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DCE	1,2-Dichloroethane
DCM	Dichloromethane
dd	Doublet of doublets
DMAP	4-(Dimethylamino)pyridine
DMC	2-Chloro-1,3-dimethylimidazolinium chloride
DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dRNase B	Deglycosylation ribonuclease B (i.e. GlcNAc-RNase B)
EDTA	Ethylenediamine tetraacetic acid
e.g.	<i>Exempli gratia</i> (for example)
Endo	Endohexosaminidase
ENGase	Endo- β - <i>N</i> -acetylglucosaminidase
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
ES	Electrospray
Et	Ethyl
<i>et al.</i>	<i>Et alia</i> (and co-workers)
Fmoc	Fluorenylmethyloxycarbonyl
Fuc	Fucose
g	Grams
GAA	Acid α -glucosidase

Gal	Galactose
Gem	Geminal
Glc	Glucose
GlcNAc	2-acetamido-2-deoxy-glucosamine
gp120	Glycoprotein 120
h	Hour(s)
H _E	Trans hydrogen
H _Z	Cis hydrogen
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum correlation
Hz	Hertz
<i>i</i>	<i>Iso</i>
IgG1-Fc	Immunoglobulin G1 fragment, crystallisable
IME	2-Iminomethoxymethyl thioglycosides
IR	Infrared
<i>J</i>	Coupling constant
kg	Kilogram(s)
L	Litre(s)
Lev	Levulinoyl
lit.	Literature
LSD	Lysosomal storage disease
μ	Micro
m	Multiplet (in NMR)
<i>m</i>	<i>Meta</i>
M	Molar
M ⁺	Molecular mass ion
Man	Mannose
mbar	Millibar
<i>m</i> -CPBA	<i>Meta</i> -chloroperbenzoic acid

Me	Methyl
mg	Milligram(s)
MHz	Megahertz
min	Minute(s)
mL	Millilitres
mol	Moles
mmol	Millimoles
mM	Millimolar
m.p.	Melting point
M6P	Mannose-6-phosphate
MPR	Mannose phosphate receptor
mol. sieves	Molecular sieves
MS	Mass spectrometry
m/z	Mass/charge ratio
ν_{\max}	Wavenumber
NCL	Native chemical ligation
NeuAc	Sialic acid (<i>N</i> -acetylneuraminic acid)
NIS	<i>N</i> -Iodosuccinimide
NMP	<i>N</i> -Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance (spectroscopy)
<i>p</i>	<i>Para</i>
Ph	Phenyl
Phth	Phthaloyl
PMP	<i>para</i> -Methoxyphenyl
ppm	Parts per million
Pr	Propyl
py	Pyridine
q	Quartet
quant.	Quantitative
R	Generic organic group, unless specified
R _f	Retention factor

rh	Human recombinant
RNA	Ribonucleic acid
RNase	Ribonuclease
rt	Room temperature
s	Singlet (in NMR)
SDM	Site-directed mutagenesis
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGP	Sialoglycopeptide
Sia	Sialic acid
S _N 2	Nucleophilic substitution, bimolecular
SPPS	Solid phase peptide synthesis
<i>t</i>	Tertiary
t	Triplet
TBAF	Tetrabutyl ammonium fluoride
Tf	Trifluoromethanesulfonyl (triflyl)
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPS	Tri- <i>iso</i> -propylsilyl
t.l.c.	Thin layer chromatography
TMS	Trimethylsilyl
triflate	Trifluoromethanesulfonate
<i>t</i> -TBP	Tri- <i>tert</i> -butylpyrimidine
UDP	Uridine diphosphate
UV	Ultraviolet
vic	Vicinal
w/v	“Weight by volume”

Amino acids:

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid

E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Chapter 1

Introduction

1.1 General introduction

Oligosaccharides / glycans are the most abundant and the most diverse biopolymers in Nature. Together with nucleic acids, proteins, and lipids, they are one of the most important types of natural organic products in living systems.¹ In contrast to the other two classes of biological polymers, carbohydrates can be highly branched, and their monomeric units can be connected to one another by linkages differing in regio- and stereochemistry. The majority of carbohydrates within cells are present as glycoconjugates, being attached to lipids to form glycolipids, or to proteins to form glycoproteins.

1.2 The biological importance of carbohydrates

Initially the roles of carbohydrates in biological systems were only thought to involve the formation of structural materials, such as chitin and cellulose, or as sources of energy, such as glucose. However, more recently carbohydrates have been found to play important roles in numerous biological events. For example, glycosylation plays a pivotal role in many key biological processes such as cell adhesion, cell-cell signaling, fertilization, neuronal development, inflammation, and the development of immune responses.¹⁻⁴ The glycosylation of proteins plays a significant role in protein folding,⁵ and in the modulation of important protein properties, such as conformation and stability,⁶⁻⁸ their susceptibility to proteases,⁶ and their circulatory lifetimes.^{7,9} Enhanced proteolytic stability as a result of glycosylation has been demonstrated, for example by comparison of RNase A, an unglycosylated pancreatic ribonuclease, and RNase B, which bears a single high mannose oligosaccharide at Asn34.¹⁰⁻¹²

In Nature glycans in glycoproteins fall into two categories: *N*-linked, in which the oligosaccharide chains are attached to asparagine residues, and *O*-linked, in which the carbohydrates are attached to serine or threonine residues. *N*-Linked glycosylation is the more common of the two. *N*-Linked glycans can be divided into three main types, each possessing a common “core pentasaccharide” structure which is attached to asparagine, and differing in the residues that extend outwards from this core: high-mannose (containing only mannose residues), complex (the core mannoses are typically further elongated with GlcNAc, galactose and sialic acid residues), and hybrid which has (at least) one antenna corresponding to each of the previous two types (Figure 1.1).

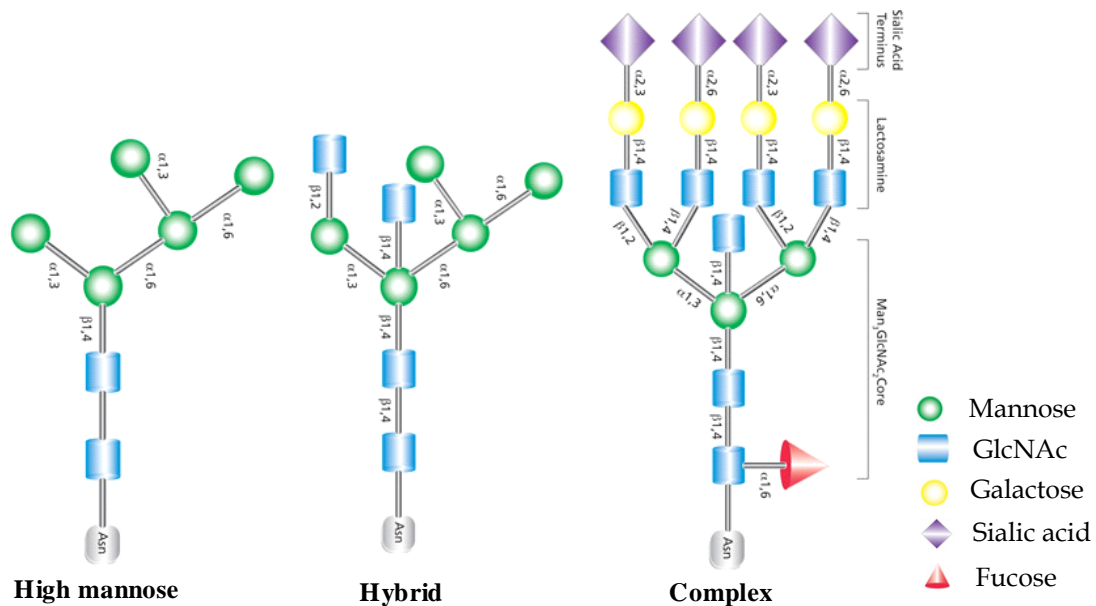


Figure 1.1 Types of *N*-linked glycan

1.3 Lysosomes and lysosomal storage diseases

Lysosomes are membrane-enclosed organelles that contain ~60 different degradative enzymes (acid hydrolases) that can hydrolyze all types of biological polymers; proteins, nucleic acids, carbohydrates, and lipids. Lysosomes function as the digestive system of the cell, serving both to degrade material taken up from outside the cell, and to digest obsolete components of the cell itself. The polypeptide chains of these lysosomal enzymes are synthesized in the rough endoplasmic reticulum where they undergo co-translational *N*-glycosylation.¹³ The resultant glycoproteins then enter the Golgi apparatus and undergo various post-translational modifications. One such post-translational modification is the attachment of mannose-6-phosphate (M6P) residues to their *N*-glycans. The transfer of proteins carrying such tagged glycans to the lysosome takes place *via* interaction with the mannose-6-phosphate receptors (M6PR).

Deficiency or loss of activity of one of the lysosomal enzymes typically leads to the accumulation of macromolecules¹³ resulting in lysosomal storage diseases (LSDs). LSDs comprise a group of more than 40 different diseases. Lysosomal storage diseases can be classified depending on the pathway affected and the nature of accumulated substrate. For example ten different enzyme deficiencies can affect the degradation of mucopolysaccharides, five defects are known in the degradation pathways of glycoproteins, one is known for the intra-lysosomal storage of glycogen, and eight deficiencies affect sphingolipid catabolism.¹⁴

1.3.1 The mannose-6-phosphate receptors

Two mannose-6-phosphate specific receptors are known.¹⁵ These receptors can be differentiated by their dependence on divalent cations.¹⁶ The cation-independent mannose-6-phosphate receptor (CI-MPR), which was initially isolated from bovine liver membranes, has a molecular mass of 215-300 kDa, and binds M6P containing ligands in the absence of cations. The cation-dependent receptor (CD-MPR), originally isolated from bovine testes and liver, has a subunit size of 41-46 kDa, and binding of M6P by the CD-MPR is enhanced by cations. Hoflack and Kornfield suggested that CD-MPR could serve as an alternate carrier for lysosomal enzymes in cells that lack the CI-MPR.¹⁵ In fact later studies have shown that the CI-MPR is multifunctional receptor;¹⁷ it can bind to proteins bearing the M6P recognition marker, as well as the peptide hormone, IGF-II. The CI-MPR also binds to M6P containing proteins that are not lysosomal hydrolases. These include transforming growth factor- β (TGF- β) precursor,¹⁸ the placental angiogenic hormone proliferin,¹⁹ the cytokine leukemia inhibitory factor²⁰ and the T-cell activation antigen CD26²¹ and many other proteins responsible for various cellular functions.^{17,22}

1.3.2 Enzyme replacement therapy for lysosomal storage disorders

The term Enzyme Replacement Therapy (or ERT) refers to a treatment using an enzyme produced by genetically engineered cells. Enzyme replacement therapy is a medical treatment involving replacing an enzyme in patients in whom that particular enzyme is deficient or absent. The concept of enzyme replacement therapy for lysosomal storage diseases was enunciated by de Duve in 1964.¹³ Initial investigations were limited by the

difficulties in extracting and purifying sufficiently large quantities of enzyme.²³ However, the development of modern molecular biology meant that lysosomal enzymes could be developed into pharmaceuticals.

In 1991, the first clinical trials for treatment of Gaucher disease with human glucocerebrosidase were reported. Gaucher disease is the most common lysosomal storage disorder. A deficiency of the enzyme glucocerebrosidase causes accumulation of the glycolipid glucocerebroside in macrophages of the internal organs like the spleen, liver, lungs, bone marrow and brain.²⁴ Various symptoms result, including skeletal abnormalities, an enlarged liver or spleen, anemia and epilepsy. Glucocerebrosidase is a glycoprotein with four *N*-glycosylation sites, three of which possess complex-type oligosaccharides.²⁵ Glycosylation plays a vital role for any ERT to be effective. They are responsible for the enzymatic activity, stability and macrophage uptake capacity.²⁶ Today ERT for type I Gaucher disease is well established, and is available commercially as imiglucerase (Cerezyme)²⁷ and VPRIV (velaglucerase alfa), recombinant human formulations produced from cultured Chinese hamster ovary (CHO) cells and modified to expose the mannose residues on glycans.

GAA (acid α -glucosidase) is a lysosomal enzyme that is involved in the normal catabolism of glycogen, and deficiency in GAA which causes Pompe disease, also referred to as glycogen storage disease type II or acid maltase deficiency.²⁸ Reduction or loss of GAA activity results in the cellular deposition of glycogen in a variety of cells, with the myocytes of the cardiac, respiratory and skeletal muscles being most severely affected. GAA has seven *N*-glycosylation sites²⁹ and its targeting from the Golgi

apparatus to the lysosome occurs *via* the M6P receptor system. Initial ERT for Pompe disease involved using human placental GAA, but was unsuccessful because this form of GAA was not phosphorylated and therefore could not be taken up by the heart and muscle cells. When GAA which possessed M6P residues was tested, it was found to be effective for the treatment of Pompe disease.³⁰ Later, Genzyme manufactured alglucosidase (Myozyme) for ERT, which is now used worldwide. Genzyme also manufacture ERT agalsidase beta (Fabrazyme) for the treatment of Fabry disease.³¹ Fabry disease is caused by a deficiency in the lysosomal enzyme, α -galactosidase A (α -galA), the enzyme responsible for the catabolism of globotriaosylceramide.³¹ Globotriaosylceramide accumulation leads to multi-organ pathology that seriously affects the kidneys, heart, and the cerebrovascular system. The initial signs and symptoms of Fabry disease emerge during childhood and adolescence, and typically include neuropathic pain crises, angiokeratomas, hypohidrosis, and gastrointestinal problems, including diarrhea, constipation, abdominal pain, and nausea and vomiting. However, because these signs and symptoms are not specific to Fabry disease, patients are frequently misdiagnosed, and the correct diagnosis may be delayed. α -galactosidase A contains three *N*-glycosylation sites, which are occupied mainly by complex and high mannose type oligosaccharides. Other ERT's that are currently available are listed in the Table 1.1.

Table 1.1

Disease	Trade Name (Generic name)	Manufacturer	Enzyme	Macromolecule	Glycosylation sites	Symptoms
Fabry	Fabrazyme (agalsidase beta)	Genzyme Corporation	α galactosidase A(homodimeric protein)	Glycosphingolipids	Three <i>N</i> -glycosylation sites (N_{139} , N_{192} , N_{215}). N_{139} links to complex type. N_{192} , N_{215} links high mannose type.	In affected males, early symptoms of the disease include acroparesthesias, angiokeratoma, and corneal and lenticular opacities. Over time the accumulation of glycolipids also leads to cardiovascular and renal disease, which ultimately leads to death in the fourth or fifth decade of life.
Type I Gaucher	Cerezyme (imigluceras)	Genzyme Corporation	glucocerebrosidase	glucosylceramide	Four <i>N</i> -glycosylation sites (N_{19} , N_{59} , N_{146} , N_{270})	Skeletal abnormalities, including thinning of bones (osteopenia), bone pain and bone fractures. Enlarged liver (hepatomegaly) or spleen (splenomegaly), or both. A decrease in healthy red blood cells (anemia) Excessive fatigue, low number of blood platelets (thrombocytopenia).
	VPRIV (velaglucerase alfa)	Shire Human Genetic Therapies, Inc.				
	Elelyso (taligluceras)	Pfizer Labs				

Glycogen storage disease type II Pompe	Myozyme (alglucosidase alfa)	Genzyme Corporation	Acid α -glucosidase	glycogen	Seven <i>N</i> -glycosylation sites (N_{140} , N_{233} , N_{390} , N_{469} , N_{651} , N_{881} , N_{924} .)	Extreme muscle weakness (myopathy), poor muscle tone (hypotonia), Enlarged liver (hepatomegaly) , heart (cardiomegaly)
	Lumizyme					
MPS I (Scheie syndrome)	Aldurazyme (laronidase)	Genzyme Corporation	α -L-iduronidase monomer of 628 amino acid residues	Glycosaminoglycans dermatan sulphate and heparin sulfate	Six <i>N</i> -glycosylation sites two of which carry the mannose-6-phosphate oligomannose oligosaccharide.	Abnormal bones in the spine, claw hand, cloudy corneas, deafness, halted growth, heart valve problems, Joint disease, including stiffness. Intellectual disability that gets worse over time, thick, coarse facial features with low nasal bridge
MPS II (Hunder)	Elaprase (idursulfase Intravenous)	Shire Human Genetic Therapies, Inc	Iduronate sulfatase	Glycosaminoglycans dermatan sulphate and heparin sulfate	Eight	Growth delay, joint stiffness, and coarsening of facial features. In severe cases, patients experience respiratory and cardiac problems, enlargement of the liver and spleen, and neurological deficits. The disorder can lead to premature death in severe cases.

MPS VI (Maroteaux-Lamy syndrome)	Naglazyme (galsulphase)	BioMarin Pharmaceutical , Inc.	Arylsulfatase B (Naglazyme)	dermatan sulphate glycosaminoglycan	Six- <i>N</i> -glycosylation sites carrying a mixture of complex, high-mannose and Four of which carry a bis-mannose-6-phosphate residue.	Maroteaux-Lamy syndrome is a progressive condition that causes many tissues and organs to enlarge and become inflamed or scarred. Skeletal abnormalities are also common in this condition. The rate at which symptoms worsen varies among affected individuals.
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1.3.3 The importance of the *N*-glycosylation of glycoproteins

As mentioned earlier glycosylation play important roles in many key biological processes. *N*-Glycosylation is a major co- and post-translational modification of proteins in all eukaryotes. Indeed it has been estimated that the majority of all secretory proteins are *N*-glycosylated. *N*-glycans play important roles in the folding of nascent polypeptides³² and play vital a role during ER-quality control processes and ERAD (endoplasmic-reticulum-associated protein degradation) of misfolded or incompletely assembled glycoproteins.³³ *N*-glycans also play a crucial role in maintaining the structure and function of the finally folded proteins.³³ In addition to the involvement in assuring the correct folding of glycoproteins, glycosylation also has other effects on the physicochemical properties of these proteins. For example, glycosylation is well known to play a role in modulating the thermostability of proteins as well as their overall charge.³⁴ *N*-Glycosylation can also play a vital role in receptor function. For example, one of the best-studied glycoproteins is the HIV viral coat protein, gp120.³⁵ The gp120 protein is integral to the initiation of contact between the HIV virus particle and its host cell by mediating the adhesion of the viral particle to the host cell surface. It is a heavily glycosylated protein; nearly half of its mass is due to the presence of 27 glycosylated residues. This protein acts as part of a co-receptor complex with the host cell CD4 protein. Association between CD4 and gp120 leads to conformational changes in these proteins that ultimately leads to membrane fusion between the host cell and the virus particle. The presence of this dense glycosylation of gp120 also acts as a natural barrier to defending immune cells and antibodies so that it is difficult for the natural immune

system to recognize and target the HIV virus for elimination. *N*-Glycosylation also plays an important role in trafficking of enzymes using different receptors. For example, the Mannose receptor (MR) is a C-type lectin, that recognizes a range of carbohydrates present on the surface and cell walls of micro-organisms.³⁶ The MR is primarily expressed on macrophages and dendritic cells and is involved in MR-mediated endocytosis and phagocytosis. The MR recognizes oligosaccharides terminating in mannose, fucose or *N*-acetylglucosamine through its C-type lectin like carbohydrate recognizing domain.³⁷ Another example is the asialoglycoprotein receptor (ASGPR). The ASGPRs are lectins which bind to asialoglycoprotein, a glycoprotein from which a sialic acid has been removed to expose galactose residues. Asialoglycoprotein is a transmembrane protein that links the cell surface to several membranous intracellular compartments. It binds galactose-terminal molecules and transports them to lysosomes inside the cell for catabolism, and is recycled.³⁶ Also, as described in section 1.3.1 mannose-6-phosphate receptors play an important role in transferring lysosomal enzymes *via* interaction with the mannose-6-phosphate receptors (M6PR).

1.3.4 *N*-Glycan biosynthesis

N-Glycan biosynthesis is a cellular process that takes place in the endoplasmic reticulum (ER) and the Golgi-apparatus, which is mediated by several enzymes, including glycosyltransferases and endoglycosidases.

The initial step of the biosynthetic pathway takes place on the endoplasmic side of the ER, and during this step, the addition of a phosphorylated *N*-acetylglucosamine to dolichol phosphate takes place.³⁸ The dolichol pyrophosphate *N*-acetylglucosamine that

is formed undergoes subsequent elongation at the *N*-acetylglucosamine unit by the addition of six additional monosaccharides (one GlcNAc and five mannoses), prior to translocation to the luminal side of the ER (Figure 1.2). Here the further addition of four more mannoses takes place, and the assembly of the precursor is completed with the linear addition of three glucose residues. The complete precursor is transferred from the dolichol-linked precursor to an asparagine side chain of the growing polypeptide chain by the action of a protein complex called oligosaccharyltransferase (OST). The next step involves removal of some of the glucose residues, and interaction of the remaining glycan with the chaperone proteins, calnexin and calreticulin, which facilitate the correct folding of the protein. When the protein is folded correctly, any remaining glucose residues, as well as one mannose are enzymatically removed from the glycoprotein prior to transfer to the Golgi apparatus for further processing by an extensive set of glycosidases and glycosyltransferases. Any misfolded proteins are transferred to the proteasome for degradation.

As a result of the differential activity of these various enzymes, glycoproteins are usually produced as complex heterogeneous mixtures in which the same protein backbone is attached to various oligosaccharide structures. These mixtures are known as glycoforms. Different protein glycoforms may have different biological functions but have almost identical in physical properties, rendering them extremely difficult to separate.¹² Access to pure homogeneous single glycoforms of glycoproteins has now become a major scientific objective.³⁹

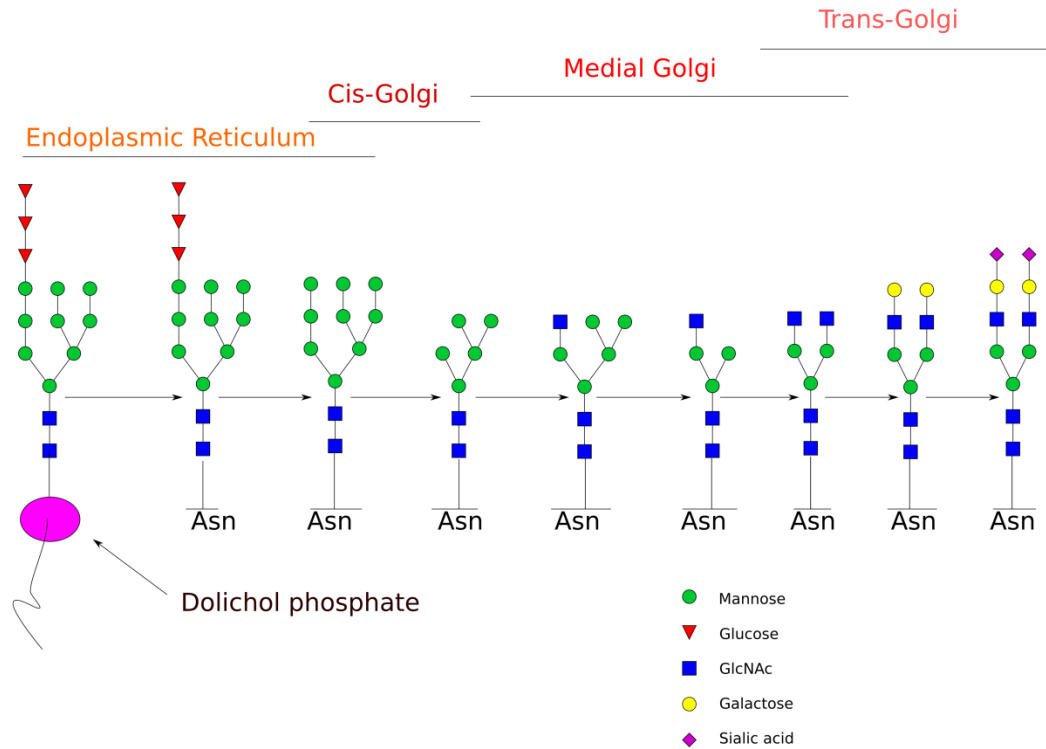
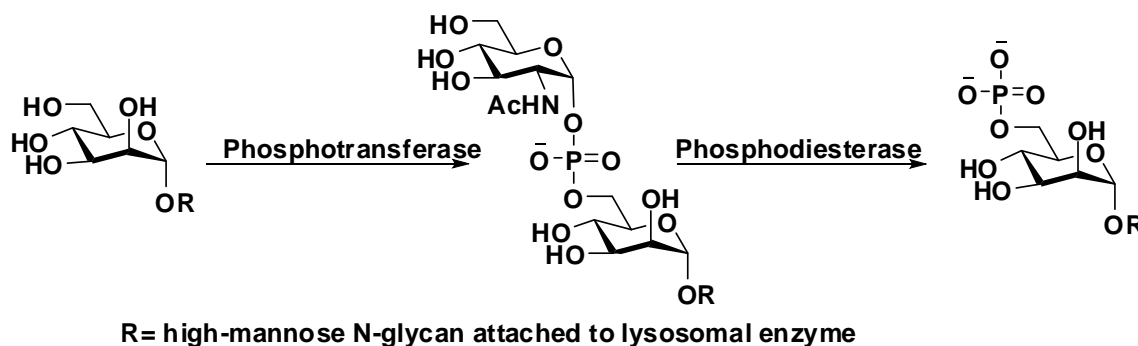


Figure 1.2 N-glycan biosynthesis

1.3.5 The biosynthesis of glycans containing mannose-6-phosphate residues

Phosphorylation of the terminal mannose residues of *N*-glycans is a post-translational protein modification that occurs *via* a two-step process involving the sequential action of two enzymes.^{40,41} In the first step (Scheme 1.1), *N*-acetylglucosamine-1-phosphate is added to the C-6-hydroxyl groups of selected mannose residues by the enzyme *N*-acetylglucosamine-1-phosphotransferase (phosphotransferase).^{40,42} Then, an “uncovering” enzyme (UCE) *N*-acetylglucosamine-1-phosphodiester α -*N*-acetylglucosaminidase, removes the GlcNAc residue to expose the mannose-6-phosphate markers.^{43,44}



Scheme 1.1

The primary purpose of the installation of these mannose-6-phosphate (M6P) residues at the non-reducing termini of high mannose oligosaccharides is to facilitate the trafficking of proteins carrying such ‘tagged’ glycans to the lysosome *via* interaction with the mannose-6-phosphate receptors (M6PR).

1.4 Previous syntheses and investigations of *N*-glycans containing M6P residues

Targeting enzymes to the lysosome *via* the M6P receptor has become an interesting area of research for the enhancement of the range of enzyme replacement therapies (ERTs) that have been developed for the treatment of LSD’s.

In order to clarify the detailed structural features required for the specific recognition by the M6PR’s, Hindsgaul and co-workers synthesized a variety of different phosphorylated oligomannosides all of which corresponded to fragments of the natural Man₉ high mannose *N*-glycan.⁴⁵⁻⁴⁷ The same group also performed a series of inhibition studies utilizing a series of chemically synthesized di-, tri-, and bi-antennary mannosides that contained M6P residues (Table 1.2).¹⁵ They performed an inhibition assay to

quantitatively probe the effect each compound had on the binding of bovine testicular β -galactosidase to each M6P receptor. They examined the effects of each of the following parameters on the binding of ligand to receptor: a) the effects of the regio and stereochemistry of glycosidic linkages between mannose residues; b) which mannose was phosphorylated in any given oligomannose sequence; c) the chain length of the oligomannose; d) the number of M6P residues per oligomannose sequence and e) the presence of an *N*-acetylglucosamine linked *via* a phosphodiester linkage to M6P.

In order to try and enhance the delivery of recombinant human acid α -glucosidase (rhGAA), Zhu *et al.* conjugated high mannose oligosaccharides bearing one or two M6P residues onto rhGAA using carbonyl-coupled hydrazine chemistry.⁴⁸ Very recently, the same group synthesized similarly modified neo-rhGAA *via* oxime chemistry,²⁸ which showed an increase in the stability of the modified enzyme without significant loss of the CI-MPR affinity.

The total synthesis of a variety of *N*-glycan structures containing M6P residues has also been reported, which were then attached to a protein *via* a non-native linker. For example Chen and co-workers successfully synthesized a bisphosphorylated mannose-6-phosphate containing *N*-glycan⁴⁹ and also an *N*-glycan carrying both mannose-6-phosphate and GlcNAc-mannose-6-phosphate motifs.⁵⁰ Furthermore, they have also synthesized several bivalent mimetic ligands (**1.1-1.4**) for the CI-MPR (Figure 1.3).⁵¹

Table 1.2

Inhibition studies of β -galactosidase binding to the CI-MPR and the CD-MPR by Ole Hindsgaul¹⁵

Inhibition of β -galactosidase binding to affinity-purified bovine testes phosphomannosyl receptors (CI-MPR and CD-MPR)					
With the exception of the lysosomal enzyme mixture containing blocked Man-6-P residues, all compounds were tested at at least five concentrations. For comparison purposes, the values presented are those that inhibited the formation of the ligand receptor complex by 50%. This concentration was determined by testing each compound over a concentration range that inhibited ligand binding to receptor between 10 and 90%. The concentration of each inhibitor that gave 50% inhibition was calculated from an inhibition plot.					
Inhibitor		Concentration at 50% inhibition		Inhibition relative to Man-6-P	
		CI-MPR	CD-MPR	CI-MPR	CD-MPR
mM					
I. Reference standard					
M-6-P ^a		0.95	0.23	1.00	1.00
II. Nonphosphorylated compounds containing Man					
M		450.00	110.00	0.002	0.002
M(α 1,2)M		11.00	3.30	0.09	0.07
M(α 1,2)M(α 1,6)	M-X ^b	>1.00	>1.00		
M(α 1,2)M(α 1,3)					
III. Di- and trisaccharides containing terminal Man-6-P residues					
P-M(α 1,3)M-X		1.70	0.35	0.47	0.82
P-M(α 1,6)M-X		2.00	0.28	0.48	0.82
P-M(α 1,2)M-X		0.36	0.043	2.64	5.35
P-M(α 1,2)M(α 1,6)M-X		0.077	0.013	13.57	17.69
P-M(α 1,2)M(α 1,3)M-X		0.065	0.014	14.62	16.43
P-M(α 1,2)M(α 1,2)M-X		0.055	0.0078	17.27	29.49
IV. Di- and trisaccharides containing monoester phosphate groups on terminal and/or penultimate Man residues					
P P-M(α 1,2)M-X P M(α 1,2)M-X P M(α 1,2)M(α 1,2)M-X P M(α 1,2)M(α 1,3)M-X P M(α 1,2)M(α 1,6)M-X		1.50	0.15	0.63	1.53
		>2.00	>2.00		
		>2.00	>2.00		
		>2.00	>2.00		
		>2.00	>2.00		
V. Biantennary oligosaccharides containing terminal Man-6-P residues					
P-M(α 1,2)M(α 1,6)	M-X	0.082	0.022	12.20	10.45
M(α 1,2)M(α 1,3)					
M(α 1,2)M(α 1,6)	M-X	0.087	0.015	10.91	15.33
P-M(α 1,2)M(α 1,3)					
P-M(α 1,2)M(α 1,6)	M-X	0.0098	0.0021	96.93	109.52
P-M(α 1,2)M(α 1,3)					
VI. Oligosaccharides containing "blocked" Man-6-P residues					
GlcNAc-P-M-CH ₃		16.00	>100.00	0.06	
CH ₃ -P-M-(lysosomal enzymes) ^c		inhibition	no inhibition		
VII. Miscellaneous					
Fructose-1-P		2.10	0.43	0.45	0.54
p-Nitrophenyl- α Man-6-P		0.79	0.15	1.20	1.53
P-M(α 1,3)M-CH ₃		1.40	0.17	0.68	1.35

^a M and PM designate D-mannose and D-mannose 6-phosphate, respectively.

^b X designates an 8-methoxycarbonyloctyl aglycone group.

^c Mixture of lysosomal enzymes from *D. discoideum*.

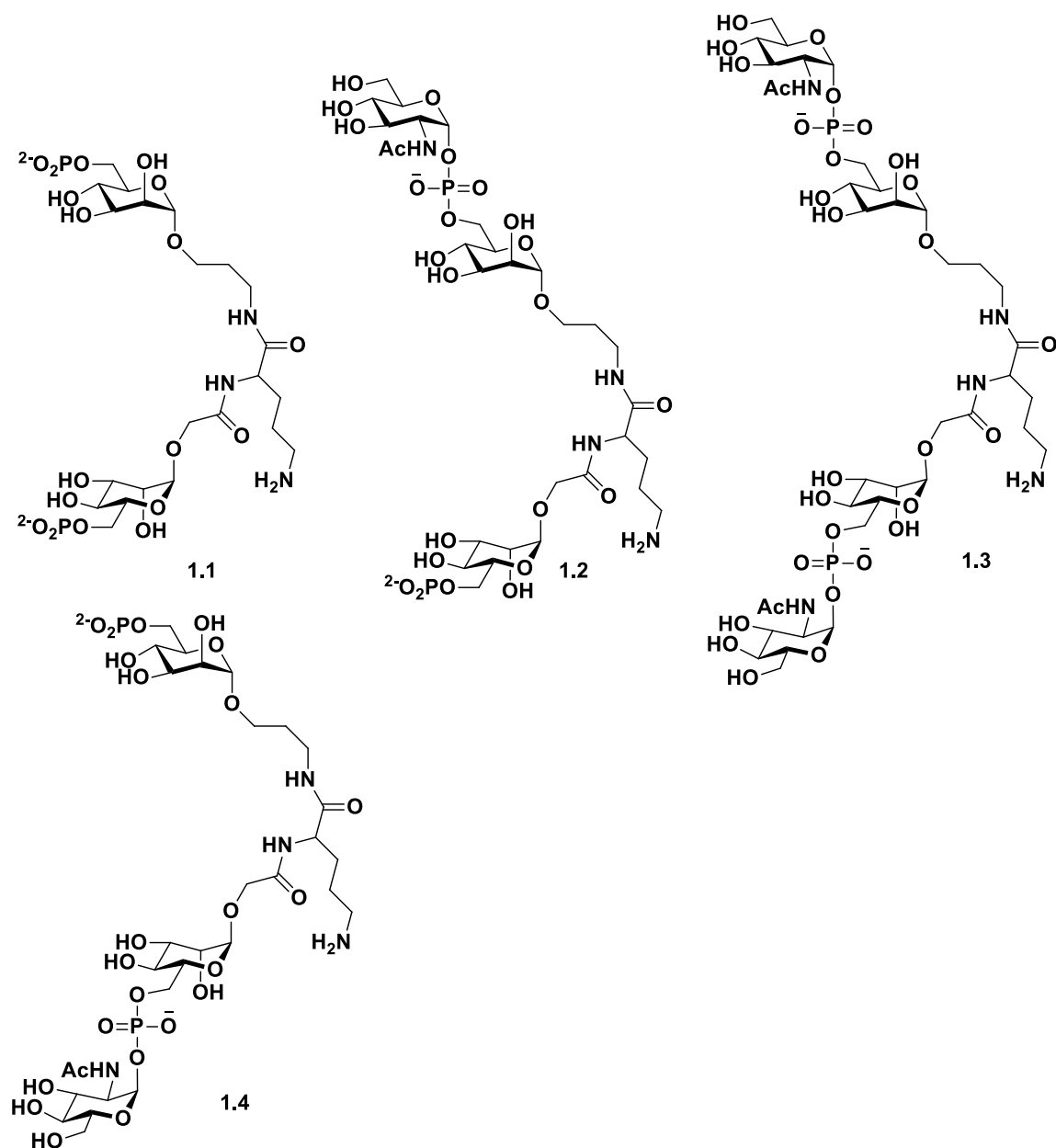


Figure 1.3 Previously synthesized bivalent mimetic ligands for CI-MPRs⁵¹

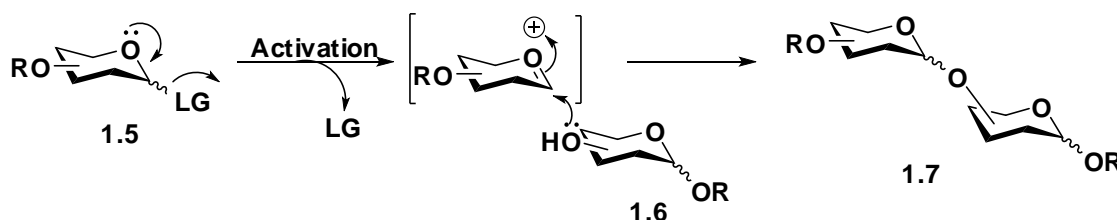
1.5 The chemical synthesis of *N*-glycans

Pure glycans of defined structure are essential research tools in glycobiology. Unlike proteins and nucleic acids, which can be obtained in homogeneous forms using biological methods such as recombinant expression and polymerase chain reaction (PCR), glycans

produced in a biological system are generally heterogeneous.⁵² Furthermore, with some exceptions like that of SGP and high mannose glycans, it is frequently difficult to obtain sufficient quantities of glycans for study from biological sources. Chemical strategies can be used to obtain homogeneous glycans in larger quantities. The chemical synthesis of oligosaccharides offers tremendous flexibility. It is possible to obtain diverse glycans, including those obtained in minute quantities from biological sources, or those for which the biosynthetic enzymes are unknown. Moreover, using chemical synthesis, the importance of different functional groups can also be tested as non-natural sugars can be introduced.⁵³

1.5.1 The glycosylation reaction

The glycosylation reaction involves the formation of glycosyl bond, a reaction first described in 1893 by Fischer.^{54,55} A ‘donor’ monosaccharide **1.5**, equipped with a leaving group at the anomeric position, undergoes reaction with a hydroxyl group of an ‘acceptor’ **1.6** (Scheme 1.2). In general a promoter is added to the reaction to facilitate the departure of the leaving group. The anomeric centre of the donor should possess a good leaving group, so that a relatively poor nucleophile, such as a hydroxyl group, can engage in bond formation.



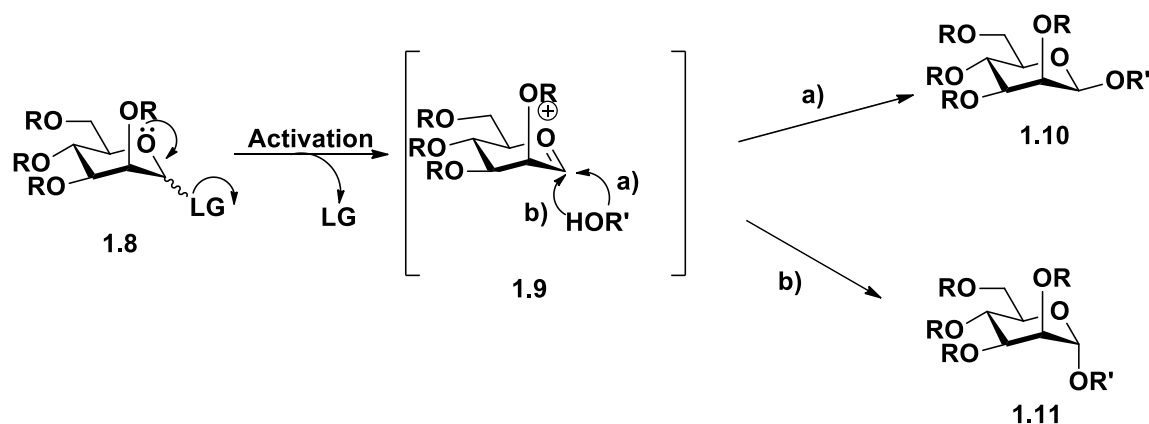
Scheme 1.2 A typical glycosylation mechanism

1.5.2 Control of regiochemistry

The inherent chemical complexities of oligosaccharides make their synthesis difficult. One of the primary challenges of glycan synthesis is the requirement of access to a single specific hydroxyl groups in presence of many others. Glycan synthesis therefore invariably requires the manipulation of various protecting groups, chemical moieties that mask other potentially reactive hydroxyl groups and prevent them from reacting to produce regioisomeric mixtures of products. The choice of protecting groups⁵⁶ and the sequence of their installation is very important for an efficient and successful synthetic route.

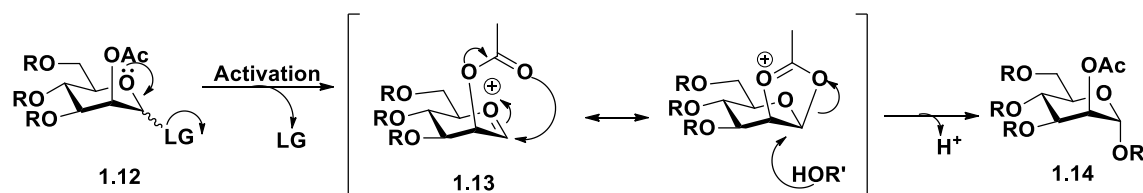
1.5.3 Control of stereochemistry

In any glycosylation reaction, the product can either be 1,2-*cis* or 1,2-*trans* with regard to the oxygen at C-2 and the oxygen of the new anomeric linkage (Scheme 1.3).



Scheme 1.3 Glycosylation to afford a) 1,2-*cis* or b) 1,2-*trans* mannosides 1.10 and 1.11

1,2-*Trans* glycosides can be synthesized using neighbouring group participation of acyl protecting groups. When an acyl group is present at the C-2 position (e.g. acetate, benzoate, phthalimide, or acetamide), it can trap the glycosyl oxocarbenium ion to form a more stable cyclic oxonium ion. With a protected glucose derivative, the 1,2-acetoxonium ion blocks nucleophilic attack of the acceptor from the α -face and results in the formation of β -glucosides. For mannose, the β -face is blocked, and α -mannosides are produced (Scheme 1.4).



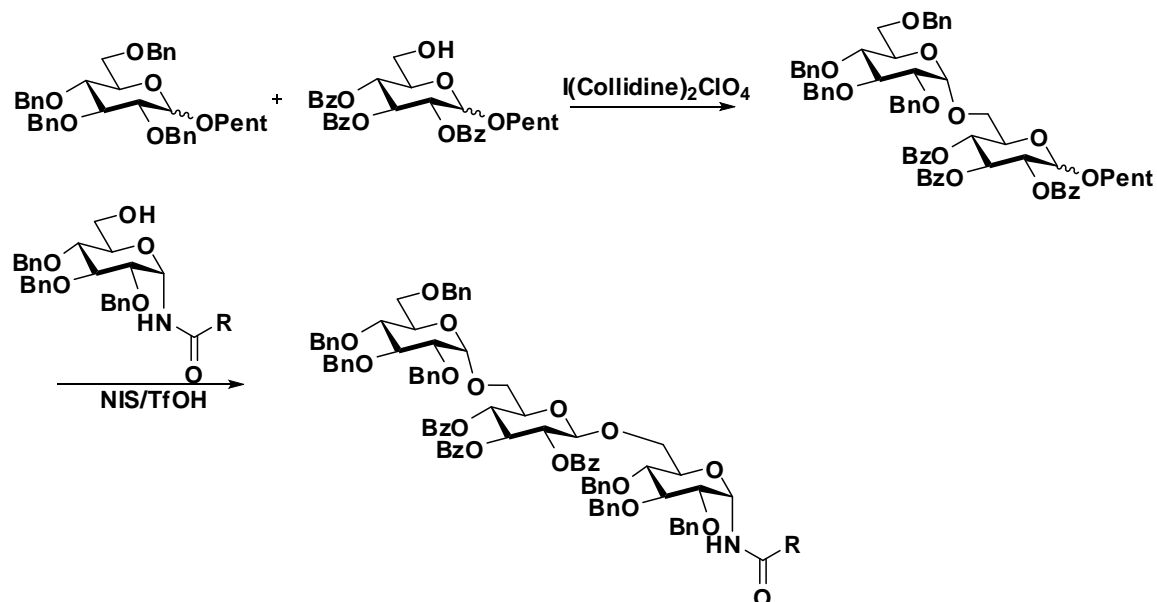
Scheme 1.4 The formation of a 1,2-*trans* mannoside by Neighbouring group participation

In contrast to 1,2-*trans* glycosides that can often be produced selectively by neighbouring group participation, the production of 1,2-*cis* glycosides requires non-participating functionalities at the C-2 position (e.g. azide, benzyl, nitrate or allyl). Even though these groups do not participate in the formation of a cyclic oxonium ion, the exclusive formation of a 1,2-*cis* glycoside is not guaranteed. One such example is the synthesis of β -mannosides, where the β face of the glycosyl donor is blocked by a neighbouring axial C-2 substituent, which disfavours attack by the acceptor from this direction. Furthermore, due to anomeric effect and intramolecular lone-pair repulsion, the α product is favored.⁵⁷

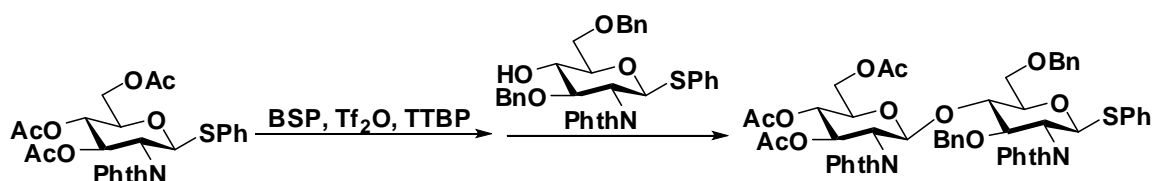
Other examples where neighbouring group participation cannot be used to form glycosidic linkages include β -rhamnosides and sialic acid derivatives. Recent developments in improved understandings of stereoelectronic and conformational effects have led to new strategies to assemble these kinds of linkages.^{58 59,60}

The synthesis of oligosaccharides usually requires purification of multiple synthetic intermediates, in addition to the final product, which is a time consuming process. Therefore, various approaches have been tried to minimize the number of purification steps. One such approach is to perform multiple glycosylation reactions in one pot,^{61,62} in which several glycosidic bonds are made without isolation or purification of intermediates. Several methods have been reported which allow multiple one-pot glycosylations.⁶³ Fraser-Reid's 'armed-disarmed' approach allows for the selective activation of an armed donor (bearing electron donating benzyl ether protecting groups) over a disarmed donor (bearing electron withdrawing ester protecting groups).⁶⁴ Treatment of a mixture of perbenzylated and per benzoylated pentenyl glycosides with iodonium di-sym-collidine perchlorate led to selective activation of the perbenzylated donor to yield a disaccharide which was then used directly in a subsequent glycosylation (Scheme 1.5).⁶⁵ A second method involves pre-activation of donors before the addition of glycosyl acceptors (Scheme 1.6).⁶² Alternatively orthogonal anomeric leaving groups can be used which can be activated using different promoters. For example Hasty *et al.* have demonstrated the elegant usage of glycosyl thioimidates as versatile building blocks in oligosaccharide synthesis.⁶⁶ They have demonstrated that glycosyl thioimidates (SBox,

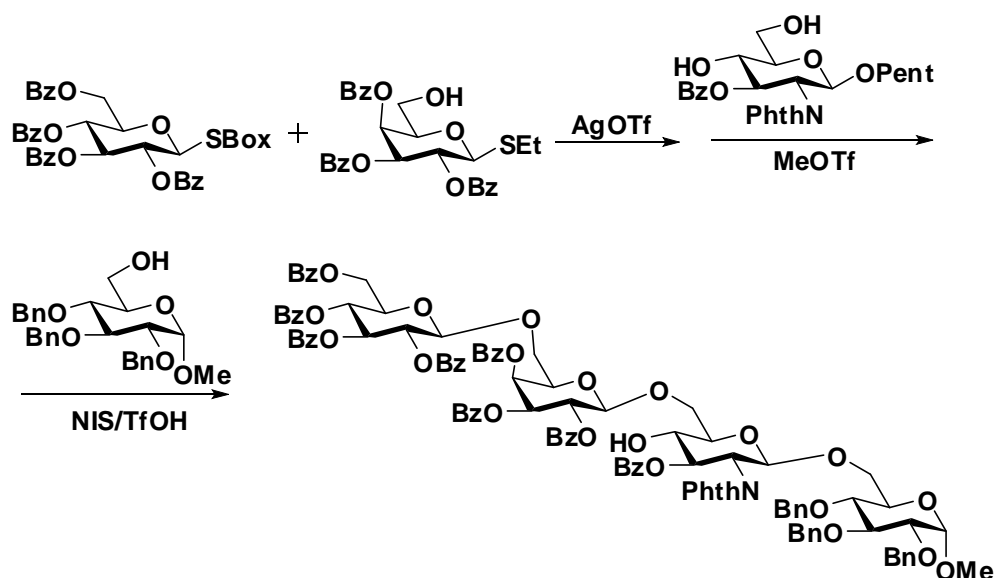
STaz, SBaz) can be orthogonally activated, making this class of donors attractive for oligosaccharide synthesis (Scheme 1.7).



Scheme 1.5 Armed and disarmed strategy⁶⁵



Scheme 1.6 Preactivation based glycosylation



Scheme 1.7 Glycosylation involving orthogonal protection

The reactivity of the donor can be modulated by a number of factors, including the leaving group, the promoter, the solvent, long-range participation effects, and steric hindrance.

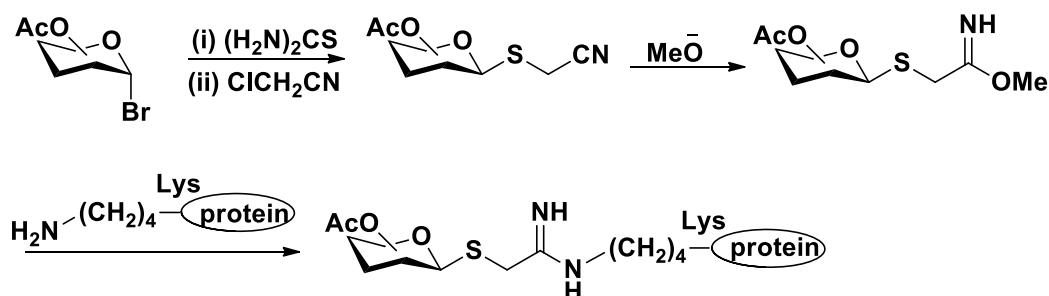
1.6 Methods of glycoprotein synthesis

As discussed earlier in the chapter, the biosynthesis of glycans is not under direct genetic control, and so glycoproteins are produced intracellularly as inseparable heterogeneous mixtures of glycoforms, in which different oligosaccharide structures are linked to the same peptide chain. Therefore, the access to pure single glycoforms of glycoproteins has become an important scientific objective in order to prepare therapeutic glycoproteins. Glycoproteins can be synthesised by various methods including chemical protein glycosylations and glycoprotein remodelling.

Chemical protein glycosylation can be divided into separate categories of indiscriminate, chemoselective, site-specific, and site-selective glycosylation.

1.6.1 Indiscriminate glycosylation

The use of 2-iminomethoxymethyl thioglycosides (IME)^{67,68} and reductive amination methods are still, after many years, the most frequently used strategies for glycoprotein preparation, both of which rely upon the high abundance of surface lysine residues. The IME method was first developed by Lee in 1976, and involves conversion of the nitrile group of cyanomethyl thioglycosides into a methyl imidate group by treatment with either sodium methoxide or methanolic HCl. These imidates can be then easily reacted with amines or protein scaffolds containing lysine residues (Scheme 1.8). For example, work by Pearce *et al.* illustrated the low, medium, and high glycosylation of lysine residues on the surface of adenoviruses.⁶⁸

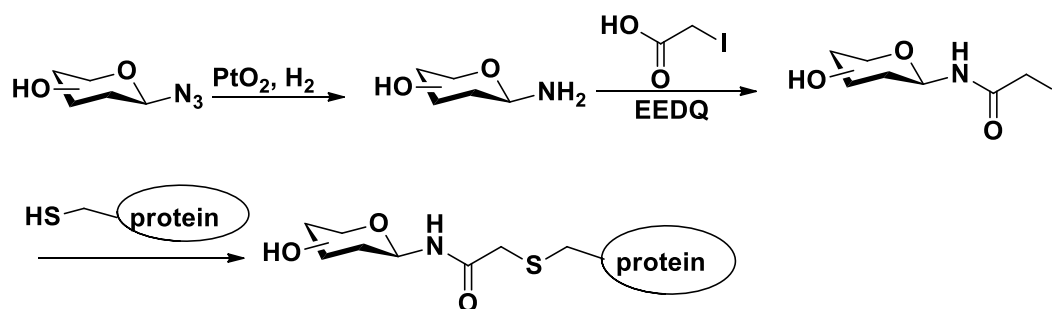


Scheme 1.8

Although these methods are useful to attach carbohydrates to proteins, due to their indiscriminate nature (all lysine residues can react), they may result in changes in protein properties and function. For example they alter the charge of the protein, which may affect its tertiary structure and activity.^{69,70}

1.6.2 Chemoselective and site-specific glycosylation

Site-specific glycosylation is different from indiscriminate glycosylation in its ability to target a specific residue in the protein. Proteins like bovine serum albumin (BSA), which contain a single free Cys, are useful substrates, since Cys is much more reactive than other amino acid residues, and can so be reacted selectively. The selective attachment of carbohydrates to proteins containing a free Cys was first reported by Davis and Flitsch using a GlcNAc α -iodoacetamide to modify a free Cys residue (Scheme 1.9).⁷¹

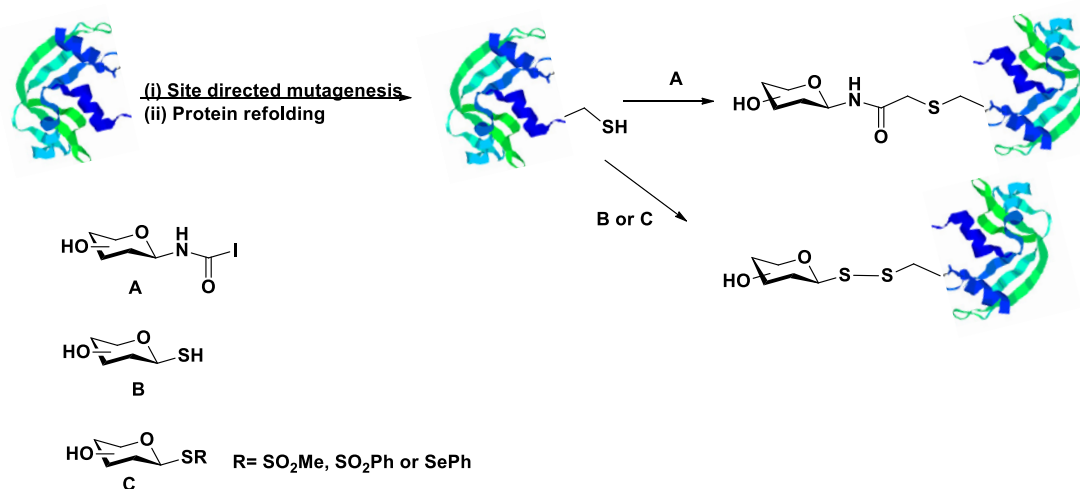


Scheme 1.9 Iodoacetamide protein glycosylation by Davis and Flitsch

1.6.3 Site-selective glycosylation

Site specific glycosylation has been extended by the use of site-directed mutagenesis to allow greater control of glycosylation. Site-selective glycosylation, as the name suggests, also allows selection of the site of glycosylation. This method involves a combination of site-directed mutagenesis and subsequent chemical modification.^{72,73} Such a strategy provides a flexible method that allows both regio- and glycan specific glycosylation of proteins. Common approaches involve the introduction of cysteine, *via* site-directed mutagenesis, as a chemoselective thiol tag to a preselected position within a given protein. The thiol can then be modified using thiol-specific carbohydrate reagents

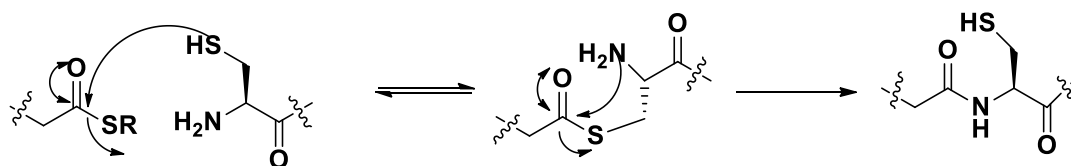
(Scheme 1.10). For example Davis and co-workers introduced a cysteine residue into a protein and the free thiol was then modified chemoselectively using electrophilic thiol-specific carbohydrate reagents, such as glycosylmethanethiosulfonates (Glyco-MTS),^{73,74} or second generation glycosyl phenylthiosulfonates (Glyco-PTS).⁷⁵ Another approach involved the selenenylsulfide (Glyco-SeS)^{76,77} mediated conjugation of sugars to cysteine residues of proteins, where pre-activation of either a cysteine mutant protein or the glycosyl thiol took place following exposure to PhSeBr. Using the glycosyl iodoacetamide approach^{78,79} Flitsch and co-workers successfully glycosylated mutants of human erythropoietin (EPO). Cysteine modification has also been exploited by Boons and co-workers⁸⁰ for the production of homogeneous disulfide linked glycoproteins by disulfide exchange reactions.



Scheme 1.10 Site selective glycosylation using A) glycosyl iodoacetamides; B) disulfides C) Glyco-MTS, Glyco-PTS and Glyco- SeS reagents.

1.7 The total chemical synthesis of glycoproteins *via* Native Chemical Ligation

The invention of native chemical ligation has revolutionized protein chemistry. Similarly, the application of NCL and its modified versions for ligation of peptides and glycopeptides provides a powerful tool for the total synthesis of large homogeneous glycoproteins.⁸¹⁻⁸⁴ Application of peptide ligation approaches for assembling synthetic peptide fragments traces its beginnings to Kemp's prior thiol capture strategy. Later, in the 1990s Kent and co-workers have demonstrated peptide ligation using Native chemical ligation.⁸⁵ NCL involves the chemoselective coupling of two protein fragments, one containing a C-terminal thioester and the other containing an N-terminal cysteine residue. The components combine to give a native peptide bond at the point of ligation (Scheme 1.11). Ligation proceeds *via* transthioesterification followed by a spontaneous and irreversible acyl shift to give a native peptide bond. The fact that this can be achieved in aqueous solution, in the absence of protecting groups, makes this a powerful technology for protein synthesis.



Scheme 1.11 Mechanism of native chemical ligation

Bertozzi and coworkers reported the first NCL-based total chemical synthesis of an 82-amino acid glycoprotein carrying two *O*-GalNAc residues: a glycosylated form of the antimicrobial protein diptericin.⁸³ Kajihara and co-workers synthesized a full size

glycoprotein carrying a complex type *N*-glycan, using two consecutive native chemical ligations of three peptide/ glycopeptide fragments followed by folding to provide the native glycoprotein.⁸⁶ Danishefsky and co-workers, described incorporation of the core pentasaccharide⁸⁷ and an epimer⁸⁸ into the same 20-residue glycopeptide and synthesis of a highly mannosylated 20-residue fragment of the HIV viral glycoprotein gp120 (Figure 1.4).^{89,90}

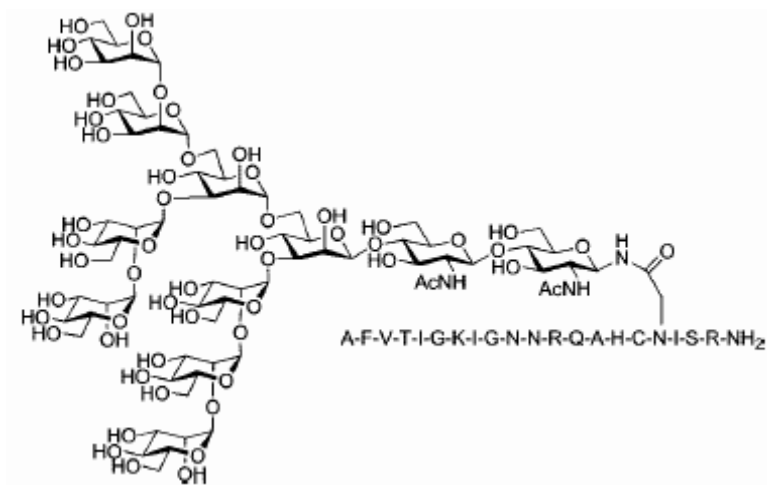
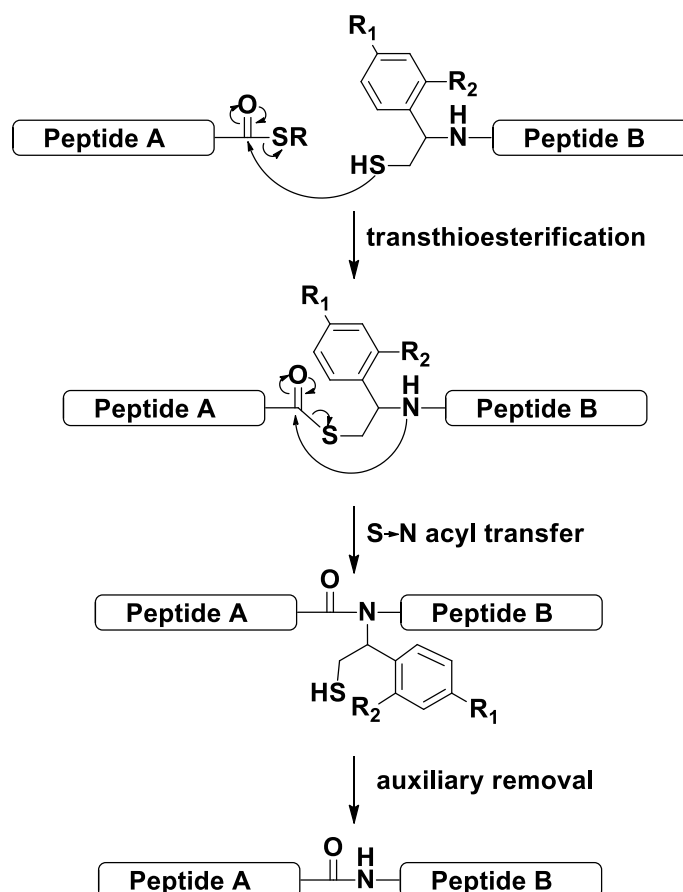


Figure 1.4 HIV viral protein gp120 fragment synthesized by Danishefsky and co-workers.^{89,90}

One drawback of NCL is the requirement for an *N*-terminal cysteine residue. Cysteine residues are not commonly found in natural peptide sequences and often have to be artificially incorporated. Several strategies have been investigated to overcome the need for a cysteine in the target at the proposed ligation site. One approach involves attaching an auxiliary thiol group to the *N*-terminal amino acid, and subsequent to ligation cleaving the auxiliary.⁹¹ Dawson and co-workers used a removable auxiliary to allow NCL

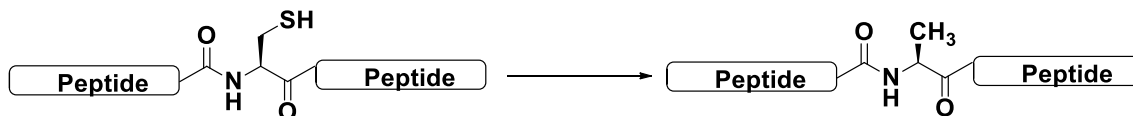
without Cys incorporation (Scheme 1.12).⁹² However, this method may not result in efficient ligation for sterically hindered amino acids. NCL was further improved by using sugar-assisted ligation (SAL),⁹³ which has the advantage over Dawson's NCL method that it is able to ligate even sterically hindered amino acids.



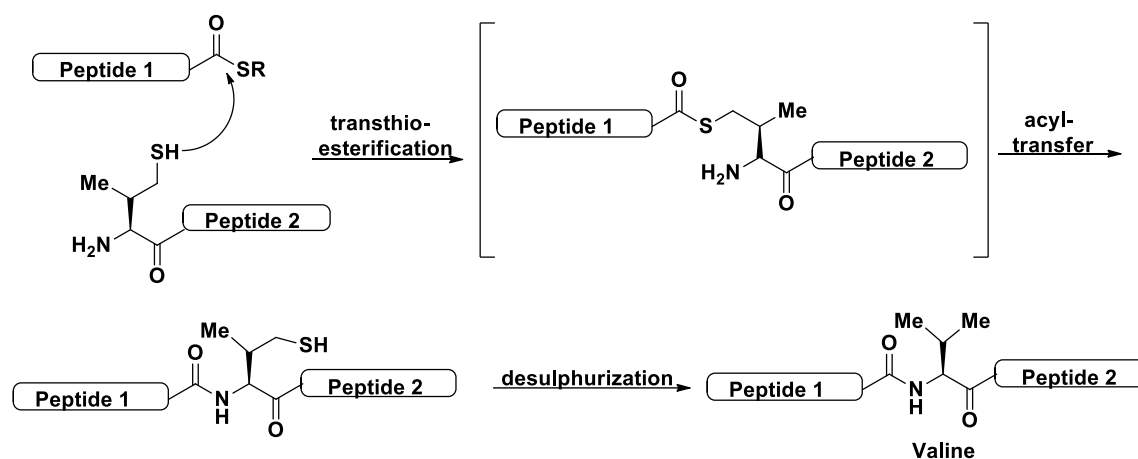
Scheme 1.12 Modified NCL, developed by Dawson *et al.*⁹²

Various other auxiliary-based strategies have been developed to mimic the cysteine-based NCL.⁹⁴ Kajihara has demonstrated ligation at a serine site by post-ligational conversion of cysteine into serine.⁹⁵ Danishefsky and co-workers have also demonstrated a mild, nonmetal-based reduction method for cysteine (Scheme 1.13), which tolerates all

thiol-containing groups, as well as oligosaccharide domains.⁹⁶ Later they have also performed native ligation at valine (Scheme 1.14).⁹⁷



Scheme 1.13 Free- radical -mediated transformation of Cys into Ala⁹⁶



Scheme 1.14 Native chemical ligation at valine⁹⁷

An array of thiolated amino acids as latent residues for Ala, Leu, Lys, Phe, Pro, Val, and Thr has been demonstrated. After ligation the thiol group was removed by radical desulfurization.⁹⁴ Recently even the synthesis of the full size erythropoietin (EPO) using alanine and cysteine based ligations has been reported by Danishefsky and co-workers.⁹⁸ EPO is a therapeutic glycoprotein that consists of 166 amino acid residues which improves the production of red blood cells, and is widely used for the treatment of anemia after chemotherapy. In their retrosynthetic design, Danishefsky's group disconnected EPO into four glycopeptide fragments (I-IV), which allowed alanine and

cysteine based ligations to reconstitute the full-length EPO (Figure 1.5). Furthermore, they also synthesized a full length native EPO carrying full size natural glycans at all four conserved glycosylation sites.⁹⁹ Although important glycoconjugates can be synthesized using NCL,¹⁰⁰ it is an extremely arduous and logistically difficult process.

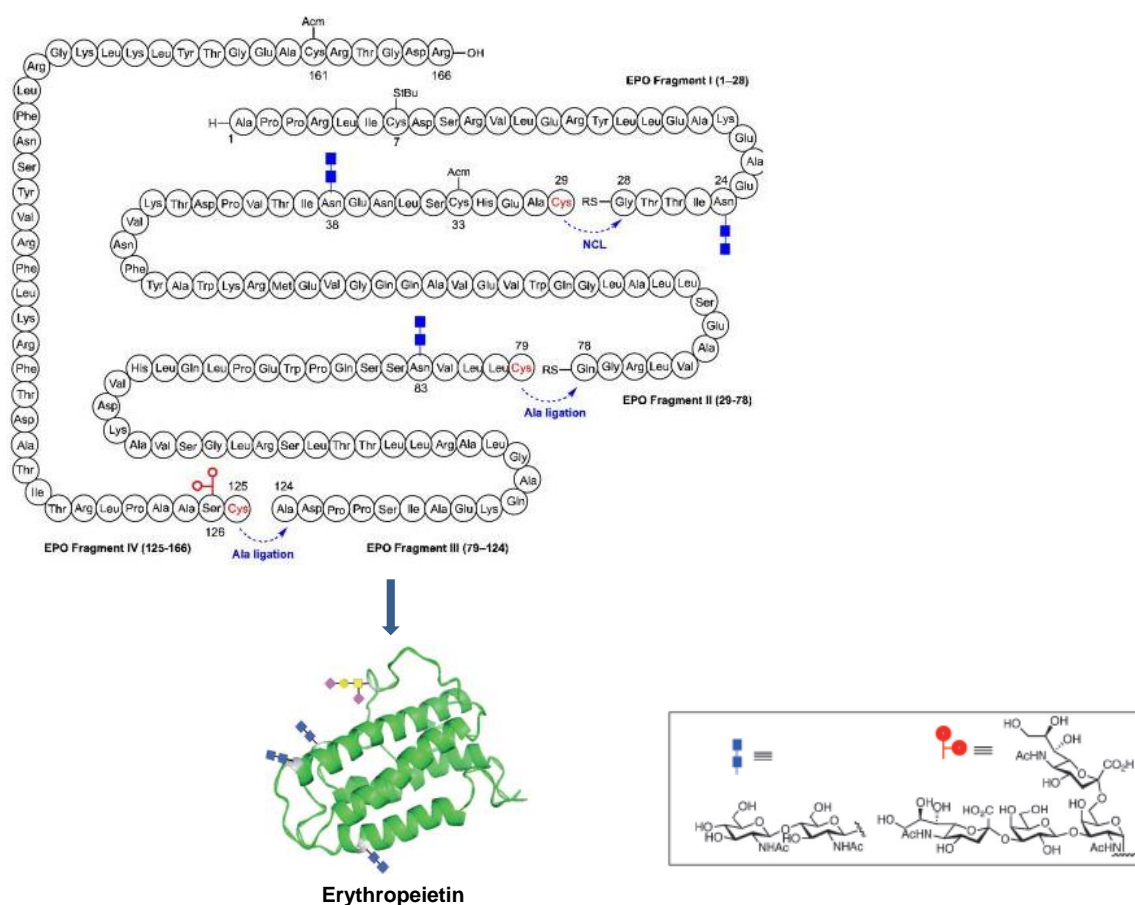


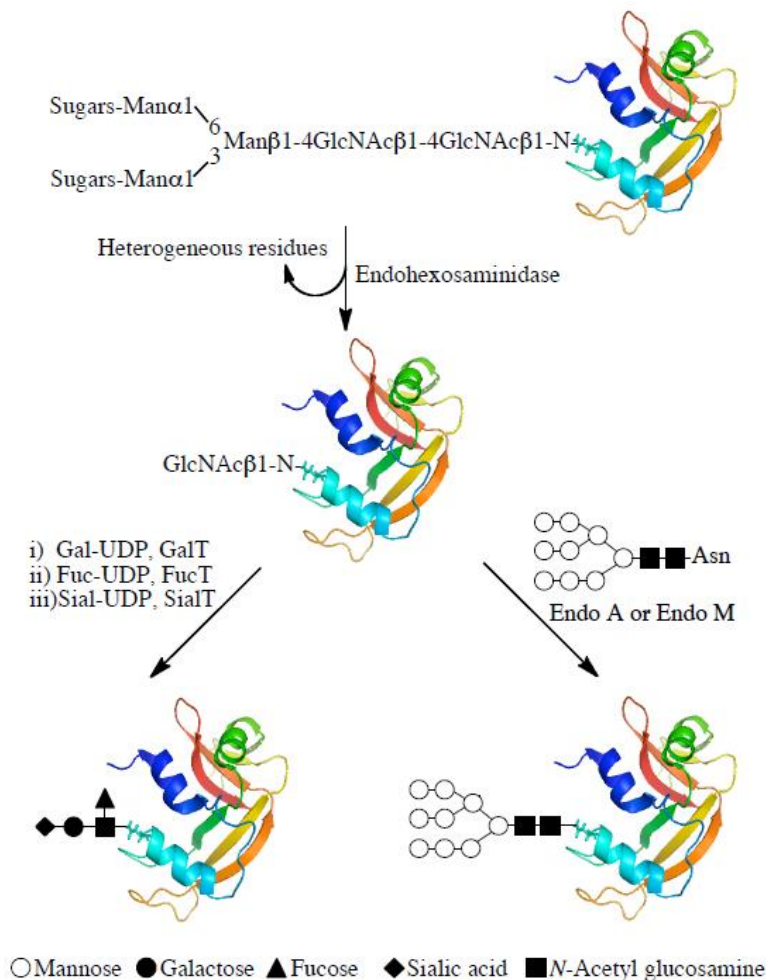
Figure 1.5 Retrosynthetic analysis of EPO by Danishefsky *et. al*^{98*}

* Adapted from Danishefsky *et. al* (98) Wang, P.; Dong, S.; Brailsford, J. A.; Iyer, K.; Townsend, S. D.; Zhang, Q.; Hendrickson, R. C.; Shieh, J.; Moore, M. A. S.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2012**, *51*, 11576.

1.8 Glycoprotein remodeling

Most recombinant therapeutic glycoproteins are currently made by over-production in CHO cells. However production in engineered CHO cells is expensive, and results in the formation of heterogeneous mixtures of glycoforms.¹⁰¹ As explained above the total chemical synthesis of glycoproteins is not only very difficult but also a very time consuming process. Therefore an efficient method which is capable of providing homogeneous glycoproteins is still needed. A number of chemoenzymatic approaches to access glycoproteins, usually involving the chemical synthesis of the glycan have been developed. The approach in which the natural glycans of a glycoprotein or glycopeptide are first trimmed back to single GlcNAc residues, which are then elaborated enzymatically, has been termed “glycoprotein remodeling”. Enzymes such as Endo H or Endo M can be used to degrade heterogeneous glycoprotein glycoforms to a produce glycoforms possessing only a single *N*-linked GlcNAc residue at *N*-linked sites. Following this, the addition of other carbohydrates may be achieved by using either glycosyltransferases or ENGases (Scheme 1.15). ENGases are enzymes which specifically cleave the chitobiose core [GlcNAc β (1-4)GlcNAc] of *N*-linked glycans between the two *N*-acetyl glucosamine residues. Glycosyltransferases can add one sugar at a time, whereas ENGases can deliver complete glycans in one step *via* either glycosylation or transglycosylation. Although glycosyltransferases have been used for the assembly of oligosaccharides¹⁰²⁻¹⁰⁵ and in some glycoprotein remodeling,¹⁰⁶⁻¹⁰⁸ their utility is limited by difficulty in accessing some of the enzymes. Furthermore, the use of glycosyltransferases is limited by their inherent specificity, their inability to attach more than one sugar at a time, and the need for complicated and expensive donors. Moreover

many of the glycosyltransferases that would be required to build N-glycan structures from a single GlcNAc are unavailable and /or would not operate in the required manner. Alternatively, the use of ENGases has allowed the conjugation of intact oligosaccharides to peptides and proteins in a single step.¹⁰⁹

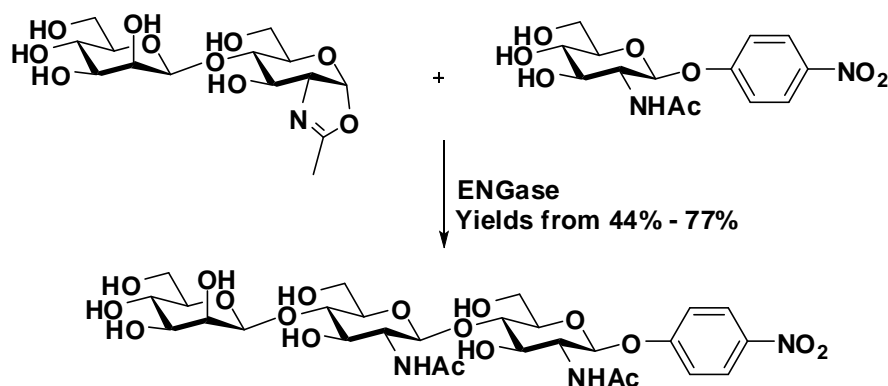


Scheme 1.15 Glycoprotein remodelling using either glycosyltransferases or endohexosaminidases[†]

[†] Adapted from Rising, T. W. D. R., D. Phil. Thesis, Oxford University, 2007.

The first example of such endo-glycosidase-catalysed transglycosylation was reported by Takegawa and co-workers for the synthesis of a Man₆GlcNAc₂-modified glycoprotein.¹⁰⁹ Subsequent investigations revealed that the enzymes of the greatest synthetic value were Endo M and Endo A. Endo M was discovered by Yamamoto and co-workers in 1988 in the culture fluid of the fungus *Mucor hiemalis* from soil.^{110,111} It was found to be capable of hydrolyzing complex, hybrid, and high mannose *N*-glycans,¹¹² and was also observed to possess transglycosylation activity.¹¹¹ Although successful, the yields for these reactions were low (5-8%) for peptide and protein remodeling¹¹³ due to competing product hydrolysis. Endo A was discovered by Takegawa, from *Arthrobacter protophormiae*, and is specific for high mannose *N*-glycans.¹¹⁴ Yields for Endo A and Endo M were initially improved by the addition of an organic co-solvent,¹¹⁵⁻¹¹⁸ but product hydrolysis still limited the efficiency of the process. Nevertheless, Endo A and Endo M catalyzed transglycosylation allowed the addition of high mannose structures and complex *N*-glycans to a variety of peptide backbones.^{111,117,119-124} For example Haneda *et al.* demonstrated the synthesis of a di-glycosylated calcitonin derivative containing two different *N*-glycans, *via* transglycosylation using both Endo A and Endo M.¹²⁵

In 2001, Shoda reported that a disaccharide bearing a 1,2-oxazoline moiety at the reducing terminus could act as a transition state mimic for some endoglycosidases, specifically Endo A and Endo M,¹²⁶ resulting in high yielding glycosylation (Scheme 1.16). In this case, the product of the glycosylation was not recognized as a substrate by the enzyme, and therefore no enzyme catalyzed hydrolysis of the product was observed.



Scheme 1.16 ENGase catalyzed glycosylation with oxazoline donors.

Later, both the Fairbanks¹²⁷⁻¹³¹ and Wang group^{121,132-139} extensively investigated the application of ENGases and further developed this methodology.

1.8.1 Work by Fairbanks *et al*

Fairbanks *et al* initially focused on investigating the substrate specificity of enzymes with regard to the donors. They synthesized a series of oligosaccharide oxazoline donors (**1.15-1.20**), corresponding to the core components of *N*-linked high mannose glycans (Figure 1.6).¹⁴⁰ Glycosylation reactions were performed with a GlcNAc-Asn glycosyl amino acid acceptor using the oxazolines as glycosyl donors catalysed by Endo M (Scheme 1.17). It was found that the minimum structural requirement was the Man β (1 \rightarrow 4)GlcNAc-oxazoline disaccharide, and the product of this glycosylation reaction was not recognized as a substrate by the enzyme, and therefore no enzyme catalysed hydrolysis was observed. However, as the oligosaccharide was increased in size, product hydrolysis was observed to become increasingly rapid.

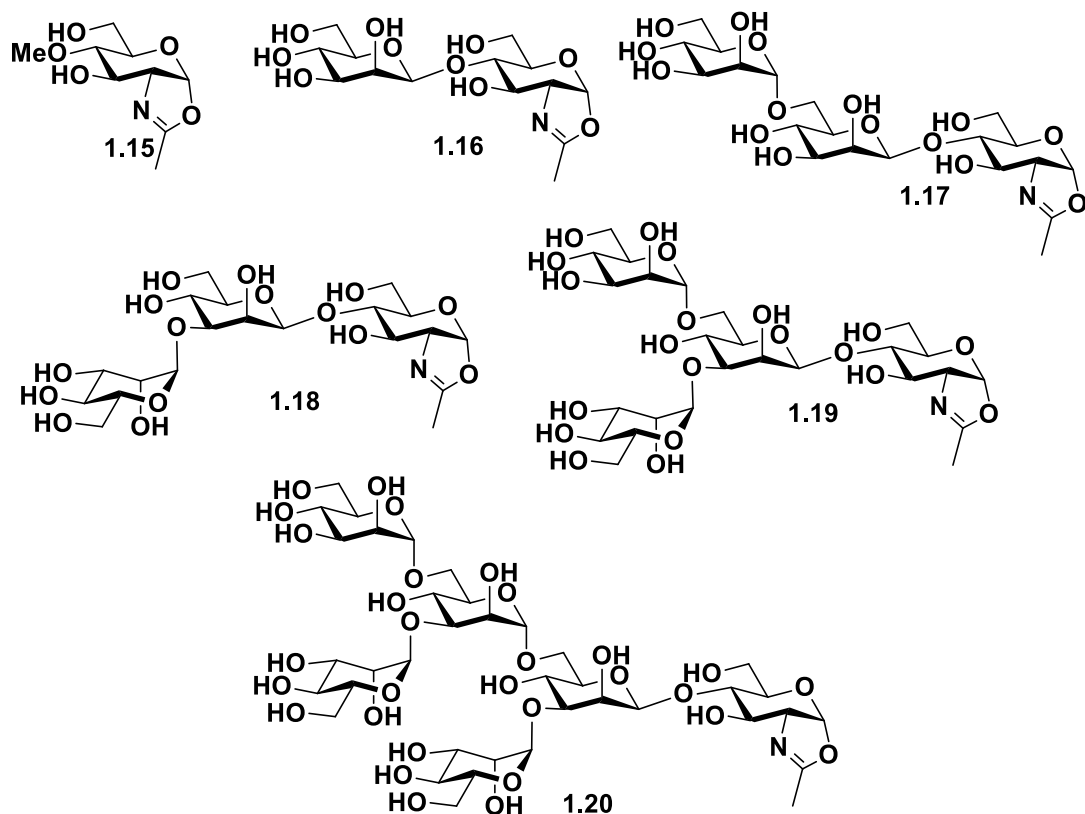
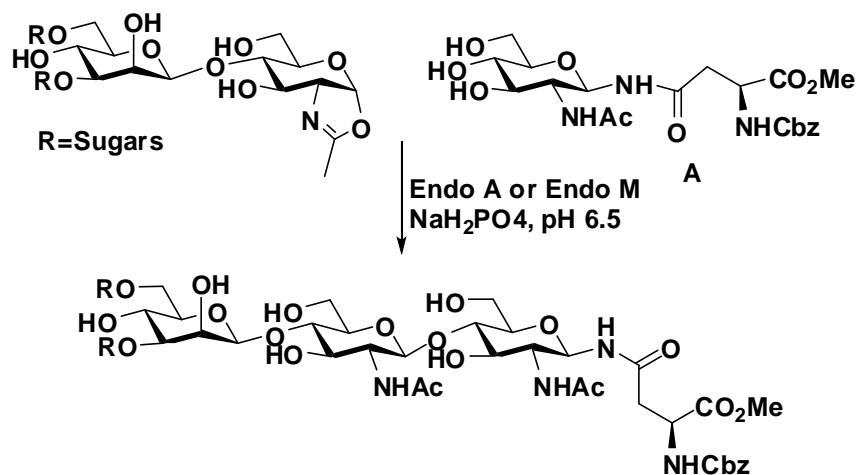


Figure 1.6 Oxazoline donors 1.15-1.20 synthesized by the Fairbanks group



Scheme 1.17 ENGase catalysed glycosylation reactions of amino acid acceptor A
with oxazoline donors

To avoid this problem, non-natural donors were synthesized (**1.21-1.26**), which were epimers of the previously synthesized *N*-glycans in which a glucose residue was incorporated in place of the mannose attached to the GlcNAc (Figure 1.7).¹⁴¹ It was found that both Endo A and Endo M were able to process these donors. In the case of Endo M, the products were not recognized as substrates and no product hydrolysis was seen, whereas Endo A was able to slowly hydrolyze the reaction products.¹²⁷ In subsequent studies they synthesized a truncated complex biantennary oxazoline¹³⁰ **1.26** and performed enzyme catalyzed glycosylations. Both Endo A and Endo M could process this donor, though only a poor yield of product was obtained.

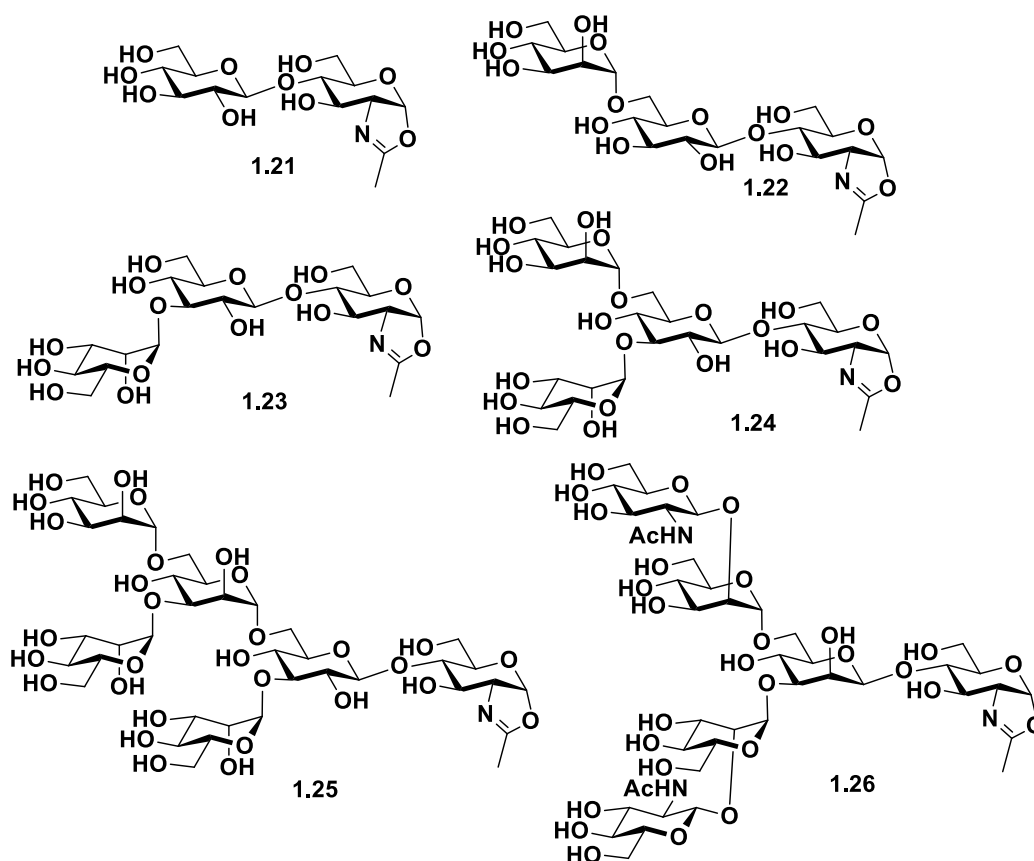
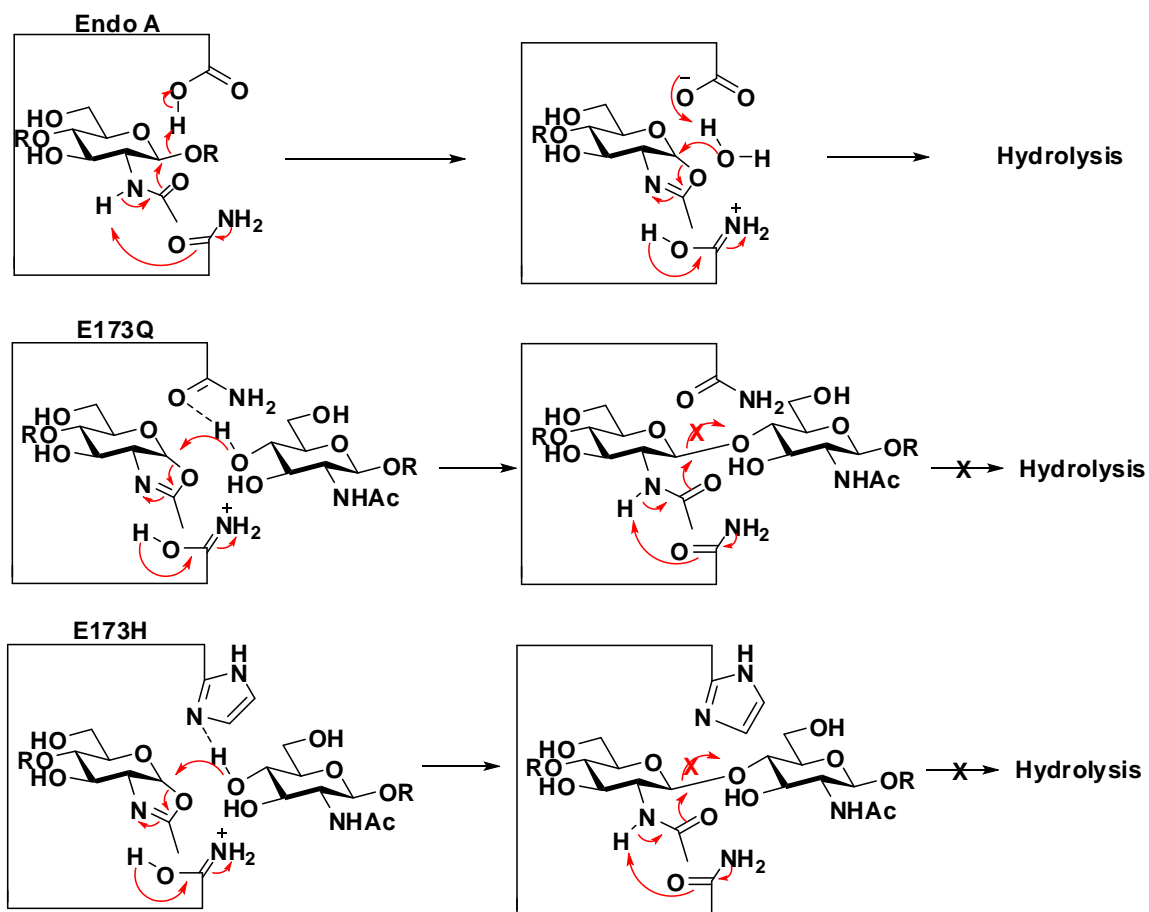
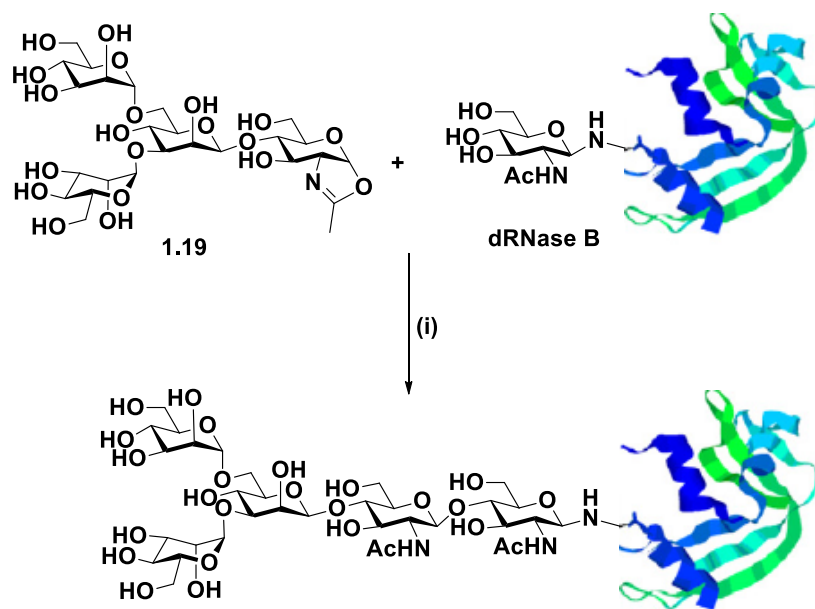


Figure 1.7 Oxazoline donors 1.21-1.26 used by the Fairbanks group

In order to decrease the amount of product hydrolysis, and to increase the efficiency of the transglycosylation, mutant enzymes were developed. During the hydrolytic mechanism of both Endo A and Endo M, a key glutamate residue in the active site acts first as a general acid to promote cleavage of the glycosidic linkage, and then, in the second step, as a general base to facilitate nucleophilic attack of water onto the oxazolinium transition structure (Scheme 1.18). Removing the general acid was expected to decrease or completely remove the hydrolytic activity of the enzyme. Fairbanks and co-workers produced two mutants of Endo A in which the key catalytic acid residue, glutamic acid 173 was replaced by glutamine (E173Q) and histidine (E173H).¹²⁹ The E173H mutant showed a slight decrease in the rate of glycosylation as compared to the wild type enzyme, together with a significantly reduced rate of product hydrolysis. The E173Q mutant showed a greater decrease in glycosylation activity, and was completely incapable of product hydrolysis. The utility of Endo A and these mutants for protein glycosylation was confirmed by carrying out the glycosylation of dRNase B using the tetrasaccharide oxazoline **1.19**. In both cases of Endo A and E173H, the enzyme was able to effect production of a single glycoform of RNase B. It was also observed that WT Endo A catalyzed the hydrolysis of the product glycoprotein, and the best yield was obtained using the E173H mutant (Scheme 1.19).¹⁴²



Scheme 1.18 Mechanism of glycosylation using oxazoline donors catalyzed by WT Endo A, and the E173Q, E173H mutants



Scheme 1.19: (i) Endo A or Endo E173H, phosphate buffer pH 6.5, H₂O, 37 °C.

More recently, Fairbanks and co-workers reported the first synthesis of glycosylated analogues of the diabetes drug pramlintide, by a combination of solid-phase peptide synthesis, and highly efficient enzymatic glycosylation using mutant enzymes.¹⁴³

1.8.2 Work by Wang *et.al*

Similarly to the Fairbanks group, Wang *et al.* also investigated the donor requirements of enzymes. They synthesized various non-natural oxazolines, including a Gal₂Man₃GlcNAc-oxazoline,¹³⁴ an octasaccharide bearing two lactose moieties, a pentasaccharide with a bisecting glucose, and a tetrasaccharide bearing azides on the 6-positions of the non-reducing termini.¹³⁷ All of these donors were found to be processed by Endo A.

Using Endo A, Wang and co-workers were also able to perform a double glycosylation of a 47-mer HIV-1 V3 domain peptide containing two GlcNAc residues,¹²¹ which demonstrated the potential application of these enzymes for *N*-glycosylation of multiple sites. This methodology was later applied in the glycoengineering of human IgG1-Fc, a monoclonal antibody with two *N*-linked glycosylation sites.¹⁴⁴

In parallel to the work of Fairbanks, which produced mutants of Endo A, Wang and co-workers generated a series of mutants of Endo M.¹³⁵ Mutants Y217F and N175A showed particularly useful activity. The Y217F mutant demonstrated enhanced glycosylation activity and a decrease in hydrolytic activity, whilst the N175A mutant showed good glycosylation activity with complete loss of hydrolysis. Using these mutant enzymes, the Wang group transferred full length glycans, such as a high mannose Man₉GlcNAc-oxazoline **1.27**, and a biantennary complex-type (SGP **1.28** from hen egg yolk),¹³⁵ to proteins (Figure 1.8).

Wang has also synthesized an array of homogeneous IgG-Fc glycoforms using the Endo A catalyzed glycosylation remodeling approach.¹⁴⁵ These results indicated that Endo A is remarkably efficient for introducing various modified *N*-glycan cores to the Fc domain by glycosylation with *N*-glycan core oxazolines. However, neither Endo A, nor the Endo M mutants (Endo M-N175A and Endo M-N175Q) was unable to efficiently transfer a full-length complex-type *N*-glycan to the Fc domain, indicating the limitations of both Endo-A and Endo-M for IgG-Fc glycosylation remodeling. Very recently, Wang and co-workers have demonstrated the remodeling of monoclonal antibodies (mAb) using

mutants of Endo S,¹⁴⁶ a member of the family GH18 hydrolases which is highly specific for the Fc region of IgGs.

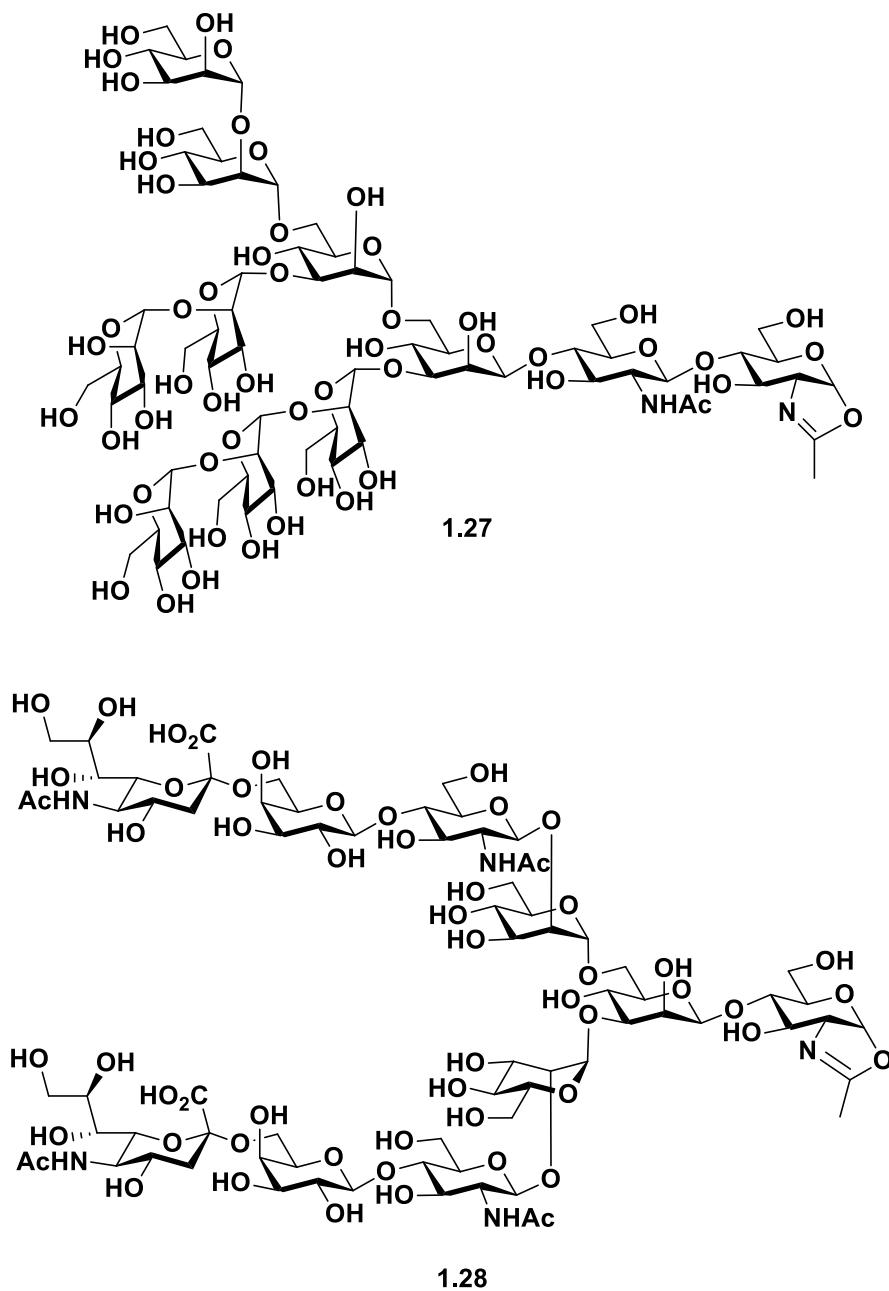


Figure 1.8 High mannose 1.27 and complex type SGP 1.28 oxazolines

1.9 Project objectives

The ultimate objective of this research program was to develop a chemo-enzymatic method to synthesise bis-phosphorylated homogeneous glycoproteins and to use them for enzyme replacement therapy.

This thesis describes the synthesis of bis-phosphorylated oligosaccharide oxazolines and investigations into their use as donors for ENGase catalysed glycosylations.

Chapter 2 describes the synthesis of a tetrasaccharide oxazoline corresponding to the core portion of *N*-glycans, which was modified by the addition of phosphate groups at the 6-positions of the two non-reducing mannose termini.

Chapter 3 details the synthesis of a hexasaccharide oxazoline, the structure of which was chosen for its reported optimal binding to the M6PR.

Chapter 4 describes a series of ENGase-catalysed glycosylation reactions that were carried out using the oxazoline donors synthesized in Chapters 2 and 3 using a GlcNAc-Asn glycosyl amino acid acceptor. Investigations into glycoprotein remodeling using de-glycosylated RNase B and partially de-glycosylated Fabrazyme are also reported.

Chapter 2

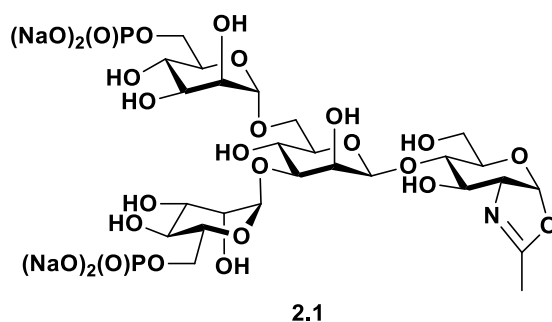
Synthesis of a phosphorylated N-glycan tetrasaccharide

oxazoline donor

2.1 Introduction

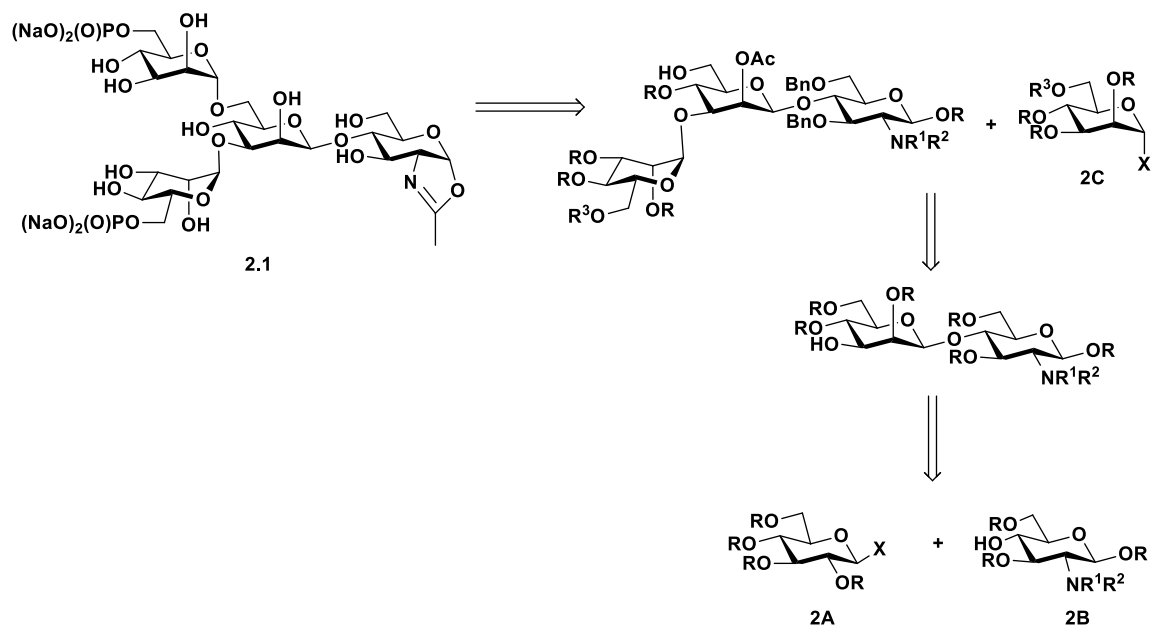
Phosphorylation of the glycan portion of glycoproteins is an important post-translational modification. Mannose-6-phosphate (M6P)-containing *N*-glycans belong to a class of high-mannose-type oligosaccharides with phosphorylation at the C6-OH group.^{147,148} These M6P *N*-glycans play an essential role in the intracellular transport of hydrolases into the lysosomal compartments in eukaryotic cells. Mannose-6-phosphate receptors (MPRs), the cation-dependent (CD-MPRs) and the cation-independent (CI-MPRs), are responsible for recognizing M6P moieties of newly synthesized hydrolases, and facilitating their transport to the lysosome.^{22,149,150} Defects in the M6P and the MPR mediated sorting system are known to result in lysosomal storage diseases (LSD).^{151,152} Understanding of the complex protein-transport system is incomplete, particularly with respect to the comprehensive structure-activity relationship of the M6P sugar-coding system at the molecular level. Not much information is available on how structure of the sugar and the position of phosphate groups on *N*-glycans affects their binding to MPRs.^{153,154} The heterogeneity of M6P-*N*-glycans and the difficulties in obtaining different, structurally well-defined M6P-*N*-glycans from natural sources impedes the ability to carry out detailed glycan receptor binding studies.¹⁵⁵ Therefore, the total synthesis of M6P-*N*-glycans with defined glycoforms would provide an attractive solution. Furthermore, it would be interesting to see if endohexosaminidases could be used to acquire homogeneous phosphorylated glycoproteins. Previous studies have shown that endohexosaminidases can recognize and process sugar oxazoline donors,^{127,128,130,132-134,136,140,156} but no investigations have been conducted to determine whether they are able to process phosphorylated sugar oxazoline donors. Therefore, in

order to carry out such an investigation, we undertook the synthesis of *N*-glycan structures containing M6P residues, and studied the ability of ENGases to catalyse their transfer to peptides and proteins. An *N*-glycan tetrasaccharide, **2.1**, was identified as a synthetic target containing two terminal mannose residues bearing phosphate groups at the 6-position. This tetrasaccharide corresponds to the core structure common in all *N*-linked glycans with the modification of phosphate groups on the C-6 hydroxyl groups of the two non-reducing terminal mannose residues.



2.2 Retrosynthetic analysis

The phosphorylated tetrasaccharide oxazoline, **2.1**, was the target of our investigation. Tetrasaccharide **2.1** could be constructed in a $(2 + 1) + 1$ approach from a core disaccharide along with a mannose donor bearing orthogonal protection at the C-6 alcohol (Scheme 2.1).



Scheme 2.1

Formation of the β -mannoside linkage, incorporation of the phosphate moieties at the C-6 position, and formation of the oxazoline were the major challenges for the synthesis of target **2.1**. The β -mannoside linkage was to be formed by the same methodology as applied previously by the Fairbanks group,¹³⁰ *via* triflation of the C-2 alcohol of a β -glucoside followed by S_N2 displacement of the triflate. Since, phosphate esters may be hydrolysed under both acidic and basic conditions,¹⁵⁷ they were to be incorporated in a late stage of the synthesis. Once the phosphate groups were installed, global deprotection would be performed before formation of the oxazoline. The oxazoline ring was to be formed using known conditions¹⁵⁸ to afford the target **2.1**.

Building block **2A** (Scheme 2.1) included 4,6-benzylidene protection and a thioglycoside at the anomeric position, since it may act as a leaving group by activation under mild conditions.¹⁵⁹ The free hydroxyls could be differentiated by regioselective alkylation at

the C-3 position¹⁶⁰ using an allyl ether, a versatile protecting group with multiple known methods of deprotection,⁵⁶ and the C-2 position could be protected with an ester that would allow formation of a β -glucoside. Levulinoyl esters were preferable as they could be selectively deprotected using hydrazine acetate in the presence of other ester protecting groups (e.g. acetate, benzoate) to allow selective access to C-2 and then subsequent epimerisation.

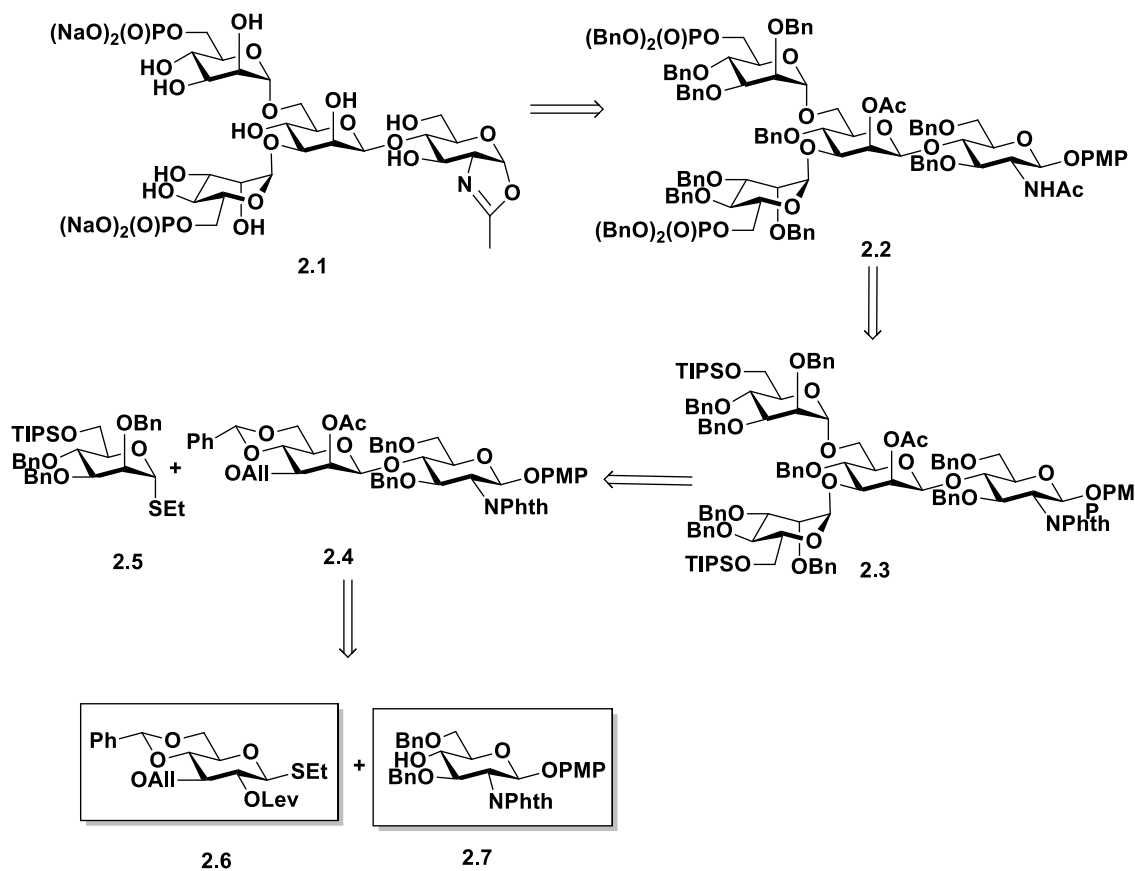
Building block **2B** required anomeric protection that would be stable towards a large number of reaction conditions, and yet which could be selectively removed in the latter stages of the synthesis. The Fairbanks group have previously employed the *para*-methoxyphenol group with success,^{127,140,141} which can be removed selectively using ceric ammonium nitrate¹⁶¹ as a single-electron oxidant. The 2-amino group was protected using a phthalimide group. Previous studies have found that the presence of *N*-acetyl glucosamine units results in low efficiency of subsequent glycosylation reactions.^{57,162} For example the acetamide could compete with the free alcohol of the acceptor and react with reactive donors, or attack the specific activator.^{23,163}

Building block **2C** required orthogonal protection at the C-6 alcohol that could be selectively removed at a late stage of the synthesis to allow installation of a phosphate group. Tri-isopropylsilylchloride was selected for C-6 hydroxyl protection. The benzyl group was selected for the protection of the phosphate groups as well as for other alcohols. A thioglycoside was chosen as a donor for the same reasons as before.

Hence, as shown in Scheme 2.2, the phosphorylated tetrasaccharide oxazoline donor **2.1** was envisioned as being derived from precursor **2.2** bearing phosphate moieties at the C-

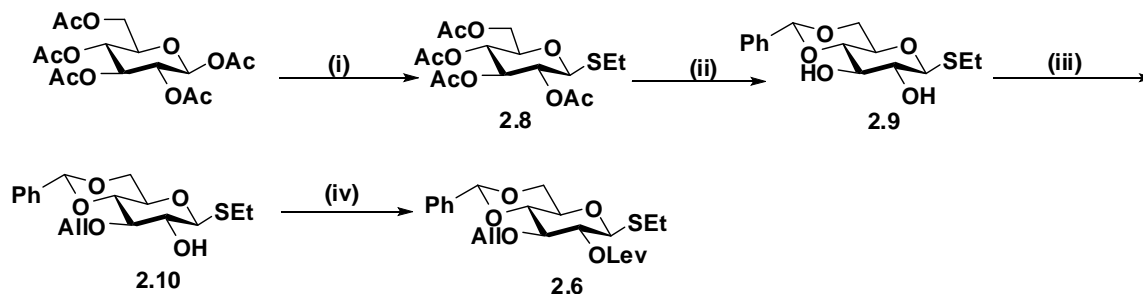
6 position of mannose, and acetate or benzyl protection of all the other hydroxyl groups. Phosphodiester **2.2** could be formed from the fully protected tetrasaccharide precursor **2.3** bearing TIPS protection at the C-6 alcohols of the terminal mannose residues. Silyl ether **2.3** could be derived from the orthogonally protected disaccharide **2.4** and the mannose donor **2.5**. Disaccharide **2.4**, with a β -mannosidic linkage could be synthesized from alcohol acceptor **2.7**, and a thioglycoside donor **2.6**.

Since removal of the phthalamide requires the use of harsh basic conditions,¹⁶⁴ this step would need to be completed prior to phosphorylation. Additionally, cleavage of the acetate in this step would not be problematic, as it would be replaced during acetylation of the amine intermediate.

Scheme 2.2. Detailed retrosynthetic analysis of tetrasaccharide **2.1**

2.3 Synthesis of building block **2.6**

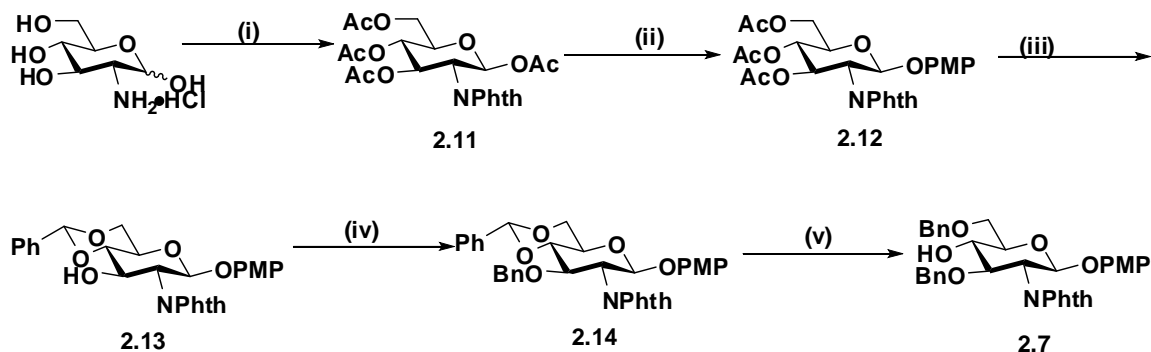
β -D-Glucose pentaacetate was treated with ethanethiol in the presence of $\text{BF}_3 \cdot \text{OEt}_2$ in DCM to give β -thioglycoside **2.8** (Scheme 2.3). Deacetylation using Zemplen conditions, followed by incorporation of the 4,6-*O*-benzylidene gave diol **2.9**. Thioglycoside **2.9** was regioselectively alkylated *via* the generation of a tin acetal *in situ* by treatment with dibutyl tin oxide followed by subsequent exposure to allyl bromide to yield alcohol **2.10**. The C-2 alcohol was protected as a levulinate ester to give **2.6**. The levulinate ester can be selectively cleaved using a non-basic reagent which does not affect the phthalimide group.



Scheme 2.3 (i) EtSH, BF₃·OEt₂, DCM, 4 h, 60%; (ii) a) NaOMe, MeOH, 1 h; b) PhCH(OMe)₂, CSA, DMF, 200 mbar, 60°C, 3.5 h, 65% over 2 steps; (iii) a) Bu₂SnO, MeOH, reflux, 1 h; b) Allyl bromide, CsF, DMF, 3 d, 63% over 2 steps; (iv) Levulinic acid, DCC, DMAP, DCM, 21 h, 84%.

2.4 Synthesis of building block 2.7

The known phthalimide protected glucosamine derivative, **2.7**, was accessed following published procedures (Scheme 2.4)^{141,165,166}

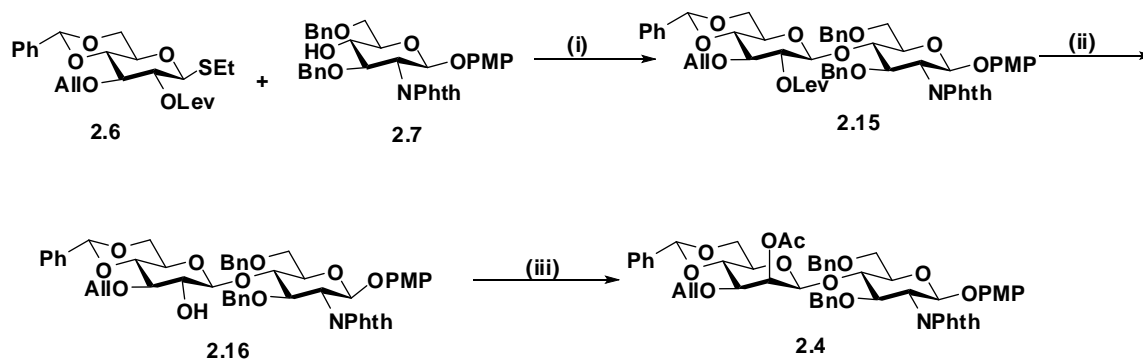


Scheme 2.4 (i) a) NaOMe, MeOH, phthalic anhydride, Et₃N, 3 d; b) py, acetic anhydride, 24 h, 50% over 2 steps; (ii) *p*-MPOH, BF₃·OEt₂, DCM, 16 h, 70%; (iii) a) NaOMe, MeOH, 50 min; b) PhCH(OMe)₂, CSA, DMF, 200 mbar, 60°C, 5 h, 54% over 2 steps; (iv) NaH, BnBr, DMF, 2 d, 83%; (v) NaCNBH₃, HCl (1M in Et₂O), THF, 0 °C to rt, 14 h, 88%.

Commercially available glucosamine hydrochloride was converted to the free base with sodium methoxide in methanol, and protected with phthalic anhydride to afford **2.11**. The hydroxyl groups were then acetylated to produce the fully protected glycoside **2.12**, which was then treated with *p*-methoxyphenol in the presence of $\text{BF}_3 \cdot \text{OEt}_2$ to afford the β -glycoside **2.12**. Zemplén deacetylation, followed by installation of a 4,6-benzylidene, gave alcohol **2.13**. The free C-3 alcohol was then benzylated to afford the fully protected glucosamine derivative **2.14**. Regioselective reductive opening of the benzylidene,¹⁶⁷ using sodium cyanoborohydride and hydrochloric acid, gave alcohol **2.7** in good yield.

2.5 Synthesis of disaccharide 2.4

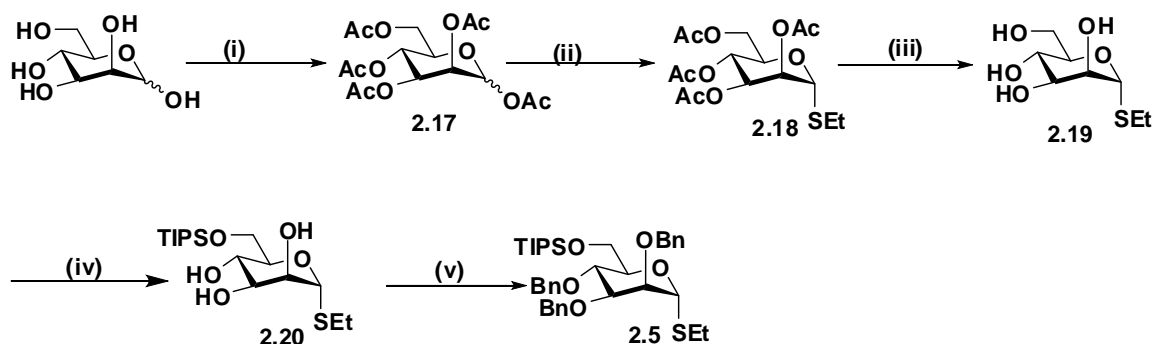
Glycosylation of thioglycoside **2.6** with the glucosamine acceptor **2.7** by activation with methyl triflate¹⁶⁸ and a hindered base (tri-*tert*-butyl pyrimidine) yielded the β -gluco disaccharide **2.15** in an excellent 91% yield (Scheme 2.5). Glycosylation occurred with complete control of anomeric stereochemistry due to neighbouring group participation by the 2-*O*-levulinoyl ester. The levulinate ester was cleaved using hydrazine acetate¹⁶⁹ to give **2.16**. The free alcohol at C-2 was epimerized by triflation followed by $\text{S}_{\text{N}}2$ nucleophilic substitution by treatment with tetrabutylammonium acetate¹⁷⁰ in toluene under sonication (following the original sonication procedure of Fürstner)¹⁷¹ to yield the β -manno disaccharide **2.4** (Scheme 2.5).



Scheme 2.5 (i) MeOTf, 3Å mol. sieves, DCM, *t*-TBP, 0 °C to rt, 15 h, 81%; (ii) H₂NNH₂·AcOH, MeOH, DCM, 21 h, 87%; (iii) a) Tf₂O, py, DCM, 0 °C to rt, 3 h; b) *N*-Bu₄NOAc, toluene, sonication, 16 h, 77% over 2 steps;

2.6 Synthesis of building block 2.5

Commercially available D-mannose was treated with pyridine and acetic anhydride to obtain mannose pentaacetate **2.17** as a mixture of anomers (Scheme 2.6). Treatment of **2.17** with ethanethiol and BF₃·OEt₂ gave thioglycoside **2.18**.¹⁷² Deacetylation using the Zemplén method afforded tetra-ol **2.19**, followed by regioselective silylation¹⁷³ gave **2.20**. Silyl protected triol **2.20** was then treated with sodium hydride and benzyl bromide to afford **2.5**.



Scheme 2.6 (i) py, acetic anhydride, 24 h, 98%; (ii) EtSH, BF₃.OEt₂, DCM, 21 h, 73%; (iii) NaOMe, MeOH, 16 h, quant.; (iv) TIPSCl, imidazole, THF, 0 °C to rt, 24 h, 89%; (v) NaH, BnBr, THF, 60 °C, 24 h, 78%.

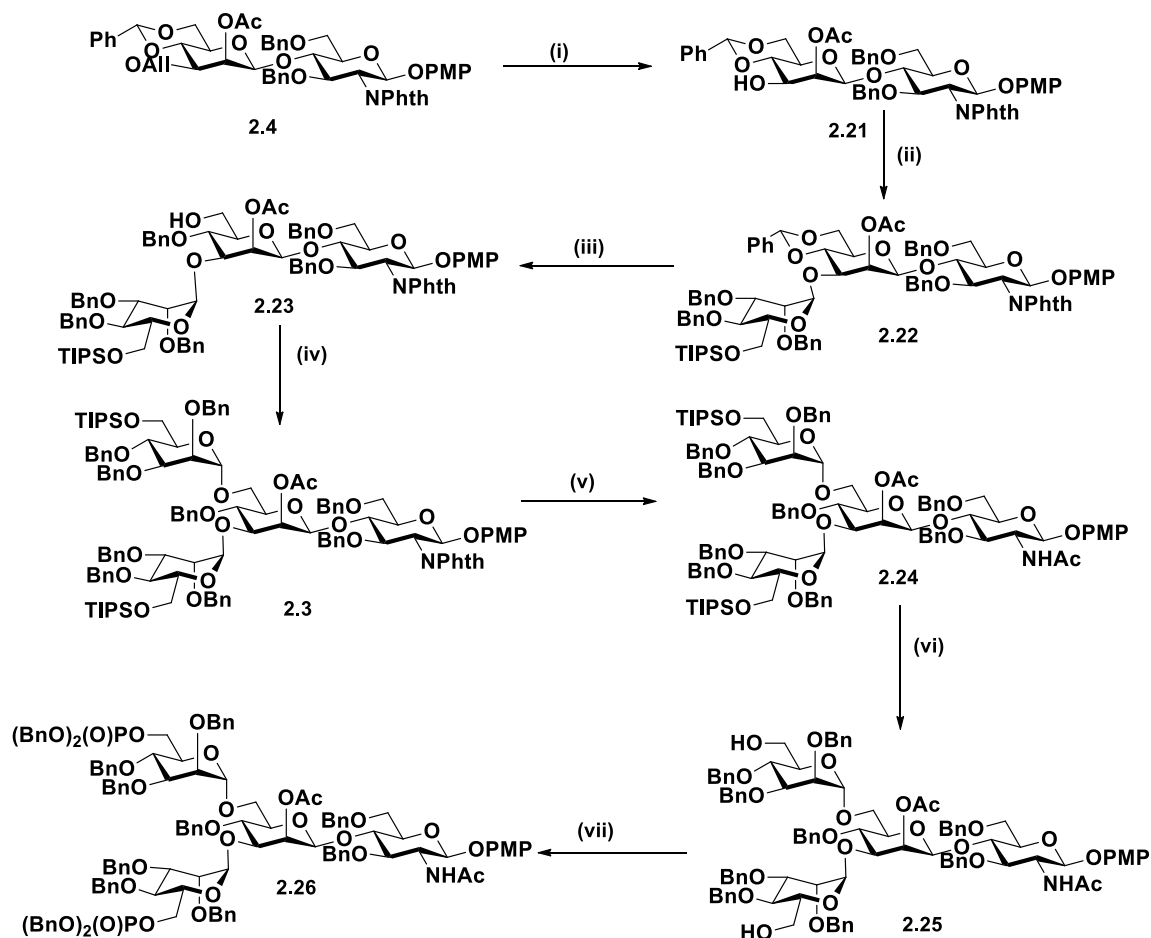
2.7 Completion of synthesis

The allyl ether of disaccharide **2.4** can be cleaved either directly, or *via* prior isomerisation of the prop-2-enyl (allyl) to the prop-1-enyl ether, which can be cleaved under mild conditions.⁵⁶ Previous studies had found the use of palladium reagents for direct cleavage of the allyl ether to be unsuccessful.²³ Catalytic palladium on carbon¹⁷⁴ produced no reaction, whilst the use of other palladium reagents resulted in cleavage of both the allyl and the 4,6-*O* benzylidene groups.

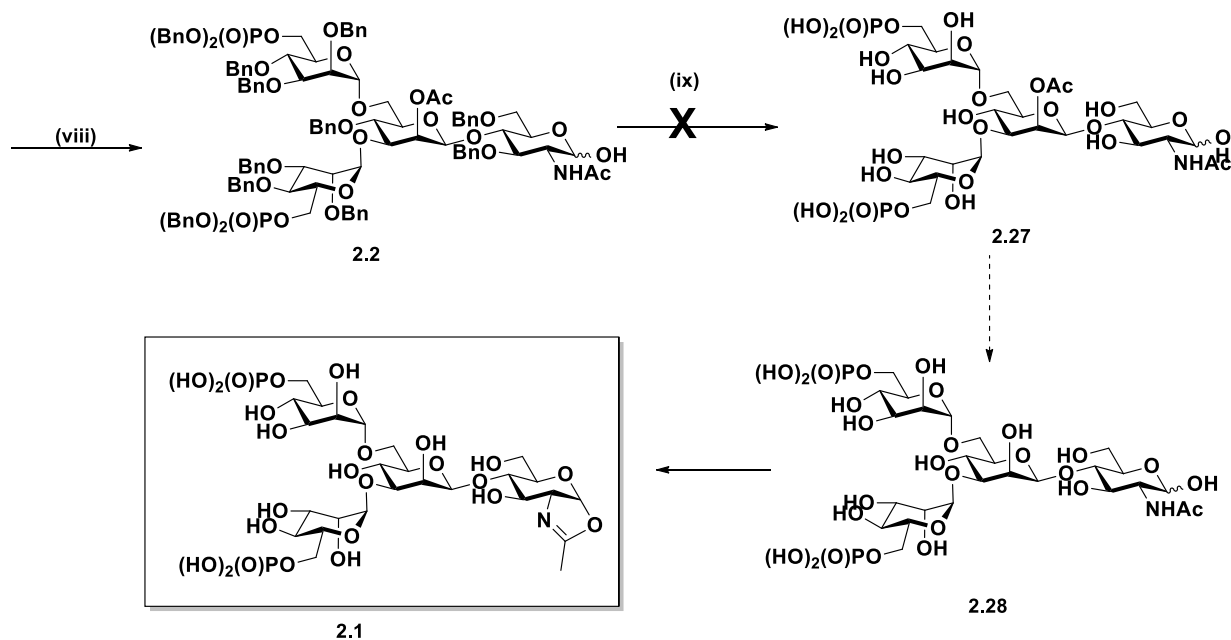
Previous students of the Fairbanks group have performed extensive investigations to find optimal methods for isomerisation of allyl to the prop-1-enyl ethers.²³ The first method investigated involved heating of the compound **2.4** with a basic catalyst,¹⁷⁵ typically potassium *tert*-butoxide in DMSO^{176,177} in which the phthalimido group was observed to undergo some ring opening. Alternatively the use of Wilkinson's catalyst, tris(triphenylphosphine) rhodium (I) chloride,¹⁷⁸ proved too harsh, and resulted in decomposition of **2.4**. The use of Grubbs second generation catalyst, 1,3-bis-(2,4,6-trimethylphenyl)-2-(imidazolidinylidene)(phenylmethylene)(tricyclohexylphosphine) ruthenium(II) chloride¹⁷⁹ also resulted in a complex mixture of products. In the end, (1,5-cyclooctadiene) bis(methyldiphenylphosphine) iridium(I) hexafluorophosphate catalyst activated with hydrogen,¹⁸⁰ gave the desired enol ether product. Treatment of the enol ether with *N*-iodosuccinimide (NIS) and water resulted in the formation of the alcohol

2.21 in excellent yield (Scheme 2.7a). Glycosylation of acceptor **2.21**, with thioglycoside donor **2.5**, was performed stereoselectively with methyl trifluoromethanesulfonate (MeOTf) and tri-*tert*-butylpyrimidine (*t*-TBP) to afford trisaccharide **2.22** in 91% yield. Regioselective reductive ring opening of the benzyldine^{181,182} using Et₃SiH and PhBCl₂ gave the free C-6 alcohol **2.23** in 94% yield. Glycosylation of **2.23** with thioglycoside **2.5** gave the tetrasaccharide core **2.3** in 79% yield, which was then subjected to treatment with ethylene diamine and methanol at reflux to remove the phthalimide protecting group. The crude amine was acetylated to afford **2.24** in 91% yield. Initial attempts to remove silyl protection using TBAF⁴⁹ as a source of fluoride proved difficult. Even after using a large excess of the reagent, only 30% product could be formed, and further increase in reagent resulted in the cleavage of the acetate group. Using BF₃·OEt₂, the TIPS group was selectively cleaved to afford **2.25** in 76% yield. Bis-phosphorylation was achieved using *N,N*-diisopropyl phosphoramidite in tetrazole (0.45 M solution in acetonitrile) followed by *m*-CPBA oxidation to give **2.26** in 58% yield (over two steps). Oxidative cleavage of the anomeric *para*-methoxyphenyl glycoside was achieved using ceric ammonium nitrate (CAN) to obtain **2.2** in 78% yield (Scheme 2.7b). Unfortunately hydrogenation reaction to remove the benzyl protecting groups of **2.2** proved to be laborious process. Due to the hindered nature of the 4-*O*-benzyl group of the central branching mannose unit cleavage of this benzyl ether by hydrogenation was extremely slow. Palladium on carbon and palladium(II) hydroxide were assayed as catalysts and were examined in both ethyl acetate and methanol as solvent. In all cases cleavage of benzyl groups required a very prolonged reaction time (15 days) and large quantities of

catalyst, which also resulted in simultaneous decomposition of the product **2.27** as well as the starting material **2.2**.

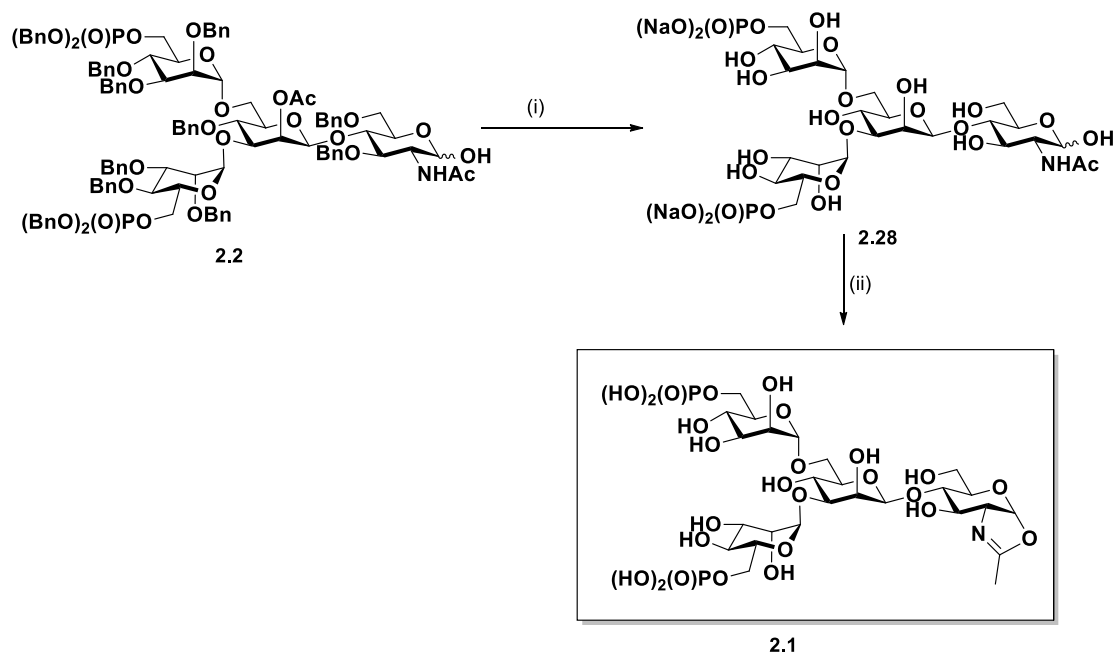


Scheme 2.7a (i) a) (1,5-cyclooctadiene) bis(methyldiphenylphosphine) iridium(I) hexafluorophosphate, THF, 14 h; b) NIS, H₂O, 18 h, 86% over 2 steps; (ii) MeOTf, 3 Å mol. sieves, DCM, *t*-TBP, 0 °C to rt, 18 h 91%; (iii) Et₃SiH, PhBCl₂, 3 Å mol. sieves, DCM, -78 °C, 50 min, 94%; (iv) MeOTf, 3 Å mol. sieves, DCM, *t*-TBP, 0 °C to rt, 18 h, 79%; v) (a) NH₂CH₂CH₂NH₂, MeOH, reflux, 18 h; (b) Ac₂O, py, 18 h, 91%; vi) BF₃·OEt₂, DCM, 0 °C, 1 h, 76%; (vii) (BnO)₂PNiPr₂, tetrazole, DCM, rt, 18 h then *m*-CPBA, -78 °C, 2 h, 58%;



Scheme 2.7b (viii) CAN, MeCN, H₂O, rt, 1 h, 78%; (ix) Pd/C, H₂, no reaction.

Eventually, an alternative strategy involving a Birch reduction was attempted, which resulting in global deprotection, i.e. debenzoylation and deacetylation of **2.2**, to afford **2.28** in 83% yield (Scheme 2.8). Deprotected phosphorylated tetrasaccharide **2.28** was then converted to the oxazoline **2.1** by treatment with DMC and triethylamine using the method published by Shoda *et al.*¹⁵⁸



Scheme 2.8 i) Na, $\text{NH}_3(\text{l})$, $-33\text{ }^\circ\text{C}$, 83%; ii) DMC, Et_3N , H_2O , rt, 30 min, 95%.

2.8 Conclusions

The target tetrasaccharide **2.1** was synthesized in 7% overall yield in 32 steps based on the longest linear sequence. The main challenges faced were the selective deprotection of the two TIPS groups and the global deprotection of the benzyl and acetate groups without decomposition of the formed tetrasaccharide.

The two TIPS groups could be selectively removed in presence of other groups using $\text{BF}_3 \cdot \text{OEt}_2$ in DCM at $0\text{ }^\circ\text{C}$. Global deprotection could be achieved using Birch condition, in which, both benzyl and acetate protecting groups can be removed in a single step in excellent yield. Furthermore, the formation of the oxazoline could be achieved easily using Shoda's method.

Hence, with the success to synthesise a bis-phosphorylated tetrasaccharide oxazoline our next aim was to synthesise further larger oligosaccharide which would resemble the natural *N*-glycan.

Chapter 3

*Synthesis of a phosphorylated N-glycan hexasaccharide
oxazoline donor*

3.1 Introduction

As discussed in the previous chapter, in eukaryotic cells, the mannose-6-phosphate receptors (MPRs) mediate the transfer of approximately 60 different newly-synthesized hydrolases to the lysosome by binding to mannose-6-phosphate (M6P) residues found on their *N*-linked oligosaccharides. Two distinct MPRs, the 46 kDa cation-dependent mannose-6-phosphate receptor (CD-MPR), and the 215-300 kDa cation-independent mannose-6-phosphate (CI-MPR), are the sole members of the P-type lectin family.¹⁷ With the successful synthesis of the phosphorylated tetrasaccharide oxazoline **2.1**, which was selected as the model for our studies, the next objective was to synthesize a full-length phosphorylated oligosaccharide. The design of the synthetic *N*-glycan (Figure 3.1) was partially based on that of the natural Man₉ structure found in the high mannose glycans of lysosomal enzymes.¹⁸³ Oligosaccharides isolated from lysosomal hydrolases have been shown to be highly heterogeneous, containing typically up to 9 mannose and 1 or 2 mannose-6-phosphate residues. Inhibition studies using synthetic phosphorylated oligomannosides have shown that linear mannose sequences of a defined chain length terminated with an α -(1,2)-linked M6P residue exhibited the greatest affinity for the receptor binding sites.¹⁵ Furthermore, bis-phosphorylated oligosaccharides exhibited increased inhibition when compared to the mono-phosphorylated oligosaccharide. The latter observation is consistent with the fact that the CI-MPR has two M6P binding sites.¹⁴⁸ On the basis of these studies, oligosaccharide **3.1** was selected as a synthetic target containing two terminal M6P residues linked *via* α -(1,2) linkage. A biantennary structure was selected for the ease of synthesis.

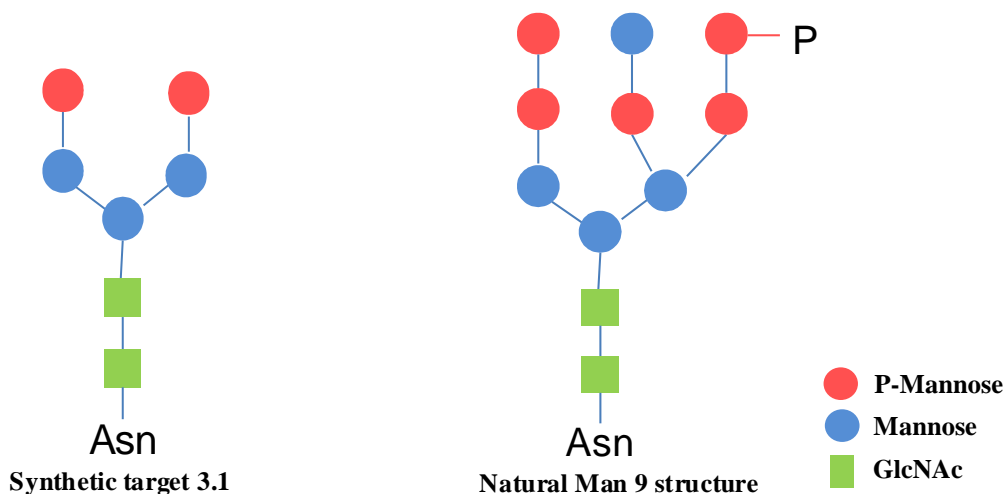
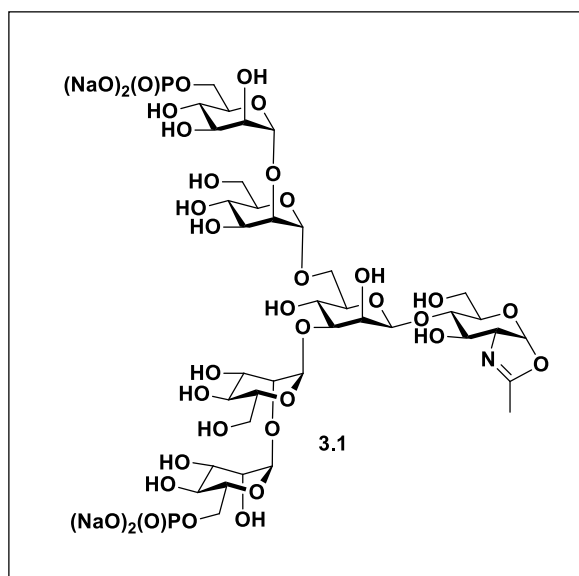
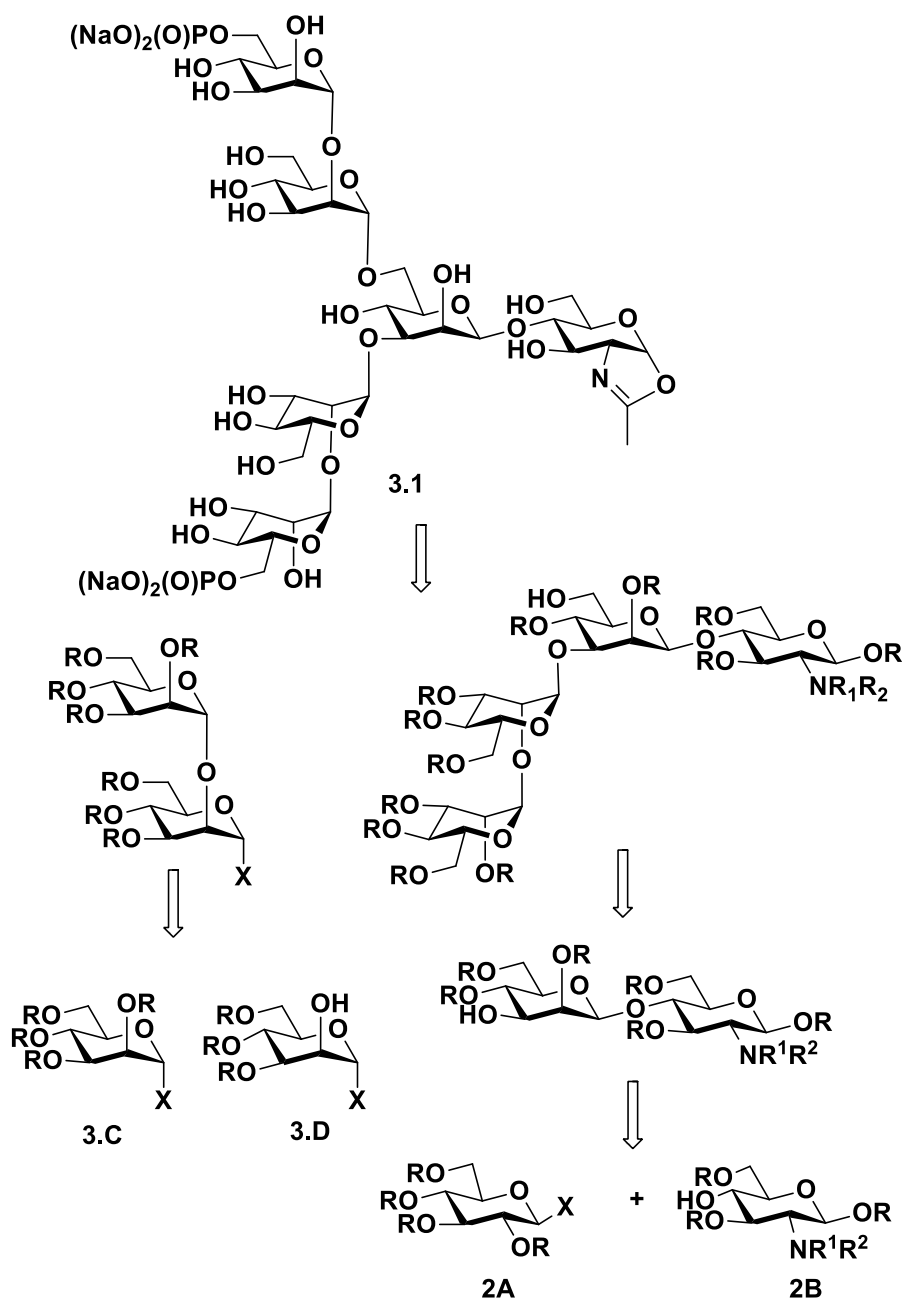


Figure 3.1:



3.2 Retrosynthetic analysis

The target of our investigation, the phosphorylated hexasaccharide oxazoline **3.1**, could be produced in a (2+2)+2 sense *via* two disaccharides that could be constructed from 4 monosaccharide building blocks (Scheme 3.1).



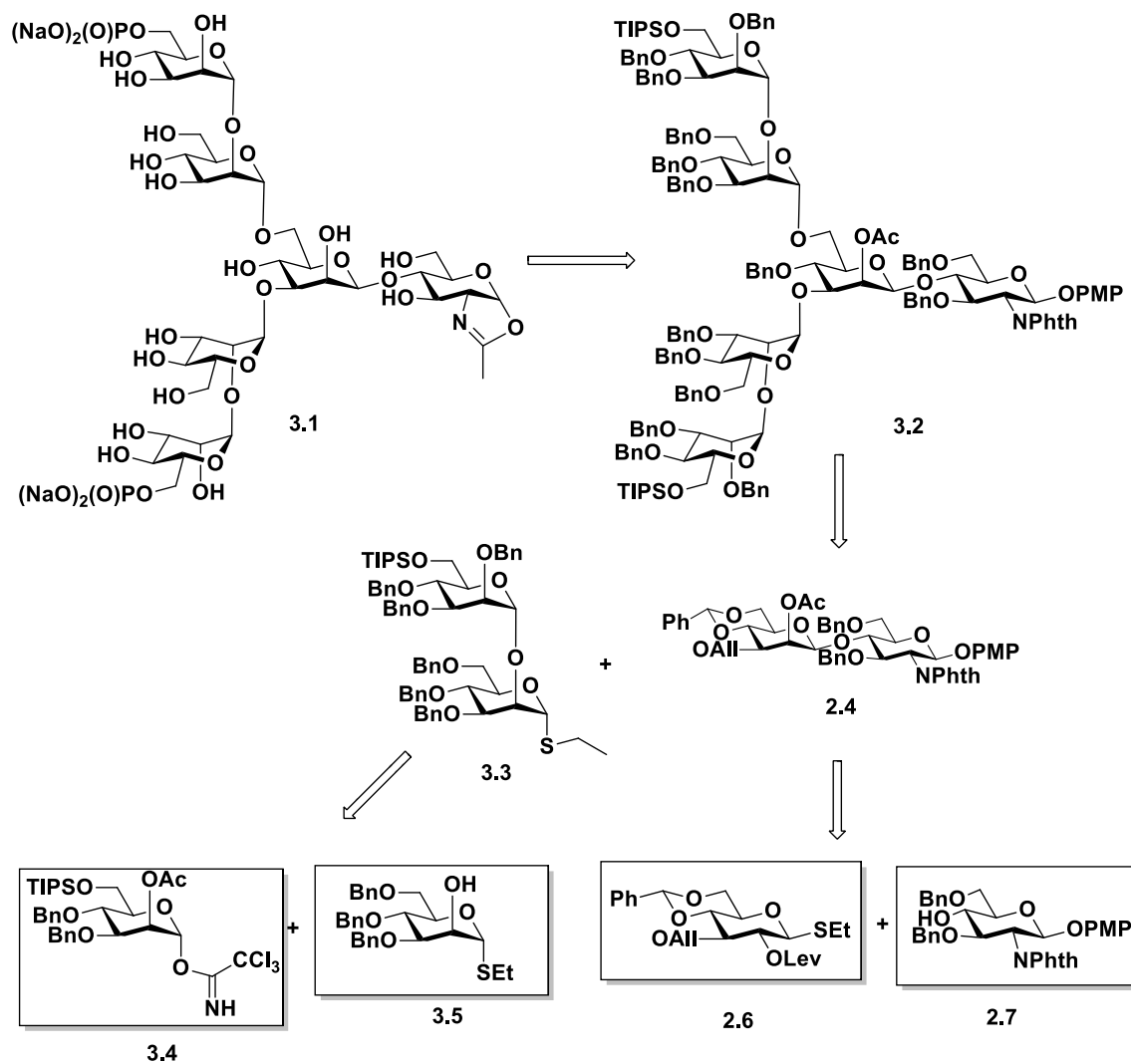
Scheme 3.1

The use of the building blocks **2.6** (**2A**) and **2.7** (**2B**), which had already been employed during the synthesis of tetrasaccharide **2.1**, would afford the disaccharide core incorporating the β -mannoside linkage (Scheme 3.1).

Building block **3C** required orthogonal protection at the C-6 alcohol that could be deprotected selectively at a late stage of the synthesis, to allow incorporation of a phosphate group. A triisopropylsilyl group was selected for C-6 alcohol protection. Benzyl groups were selected for the protection of the C-3 and C-4 alcohols. Acetate was selected as a protecting group for the anomeric position as it could be easily deprotected and replaced with a leaving group, and could be accessed from mannose pentaacetate on large scale. Acetate protection was also used at C-2 to allow for α -mannoside formation *via* anchimeric assistance.

Building block **3D** was prepared by the formation of an orthoester with subsequent ring opening to introduce ethanethiol at the anomeric position.¹⁸⁴ A thioglycoside was chosen as the anomeric leaving group because it could be activated under mild conditions.

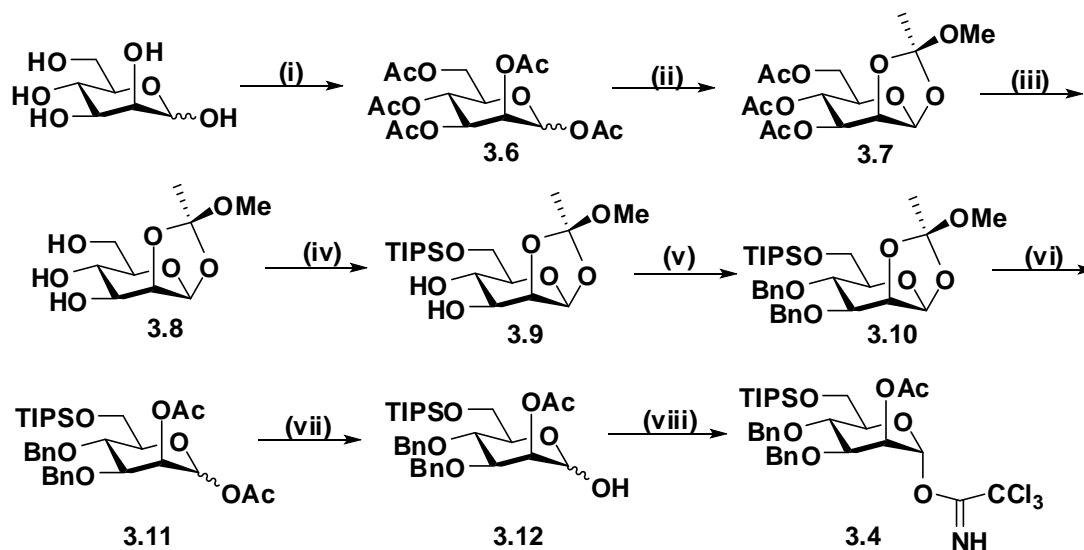
Thus, hexasaccharide **3.1** was visualised as being derived from precursor **3.2** bearing phosphate moieties at the C-6 positions of the two mannose units and acetyl and benzyl protection on all the other groups. Hexasaccharide **3.2** could be accessed from disaccharide donor **3.3** and the orthogonally protected disaccharide **2.4** (Scheme 3.2). Donor **3.3** could be synthesised from the imidate donore **3.4** (building block 3C) and the *manno* acceptor **3.5** (building block 3D). Finally it was envisaged that the disaccharide **2.4** could be synthesised from the alcohol acceptor **2.7**, and a thioglycoside donor **2.6**.

Scheme 3.2 Detailed retrosynthetic analysis of hexasaccharide **3.1**

3.3 Synthesis of building block **3.4**

Building block **3.4** was synthesized following Hendrickson's method.¹⁸⁵ Mannose was peracetylated to afford pentaacetate **3.6**, which was then treated with 33% HBr in acetic acid to give the α -bromide. The glycosyl bromide was then treated with 2,6-lutidine in a mixture of methanol and DCM to afford the orthoester **3.7** in good yield (Scheme 3.3). Zémpfen deacetylation afforded triol **3.8**, which was followed by regioselective silylation

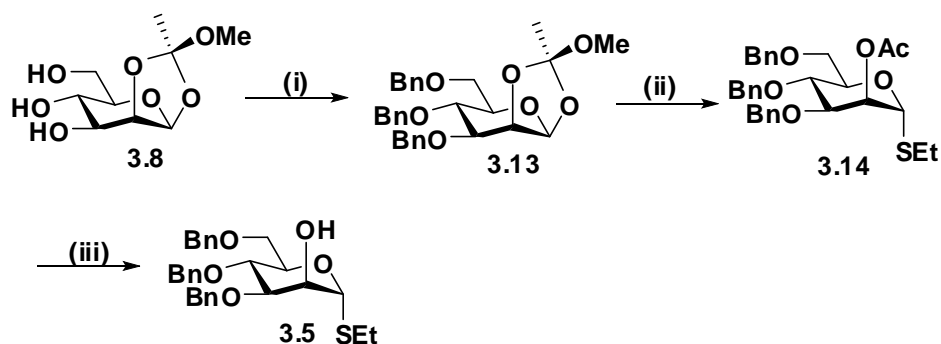
to give **3.9**. The TIPS protected orthoester was then treated with benzyl bromide and sodium hydride to afford the benzylated orthoester **3.10**. It was found that acid catalysed regioselective opening of orthoester **3.10** gave a mixture of C-1 and C-2 acetylated products. To overcome this problem, the orthoester was then exposed to acetic anhydride in pyridine to give **3.11** as the sole reaction product. The anomeric acetate was then selectively deprotected using hydrazinium acetate in DMF to afford hemiacetal **3.12**. Trichloroacetimidate **3.4** was then prepared by reaction of alcohol **3.12** with trichloroacetonitrile and DBU in DCM (Scheme 3.3)



Scheme 3.3 (i) py, acetic anhydride, 24 h, 98 %; (ii) a) HBr, DCM, 2 h, 0 °C; b) 2,4,6-collidine, MeOH, 24 h, 50 °C, 85% (over 2 steps); (iii) NaOMe, MeOH, quant; (iv) TIPSCl, imidazole, THF, 0 °C to rt, 24 h, 48%; (v) BnBr, NaH, DMF, 24 h, 92% (vi) a) 80%, aq AcOH, 18 h, b) py, acetic anhydride, 24 h, 68%; (vii) hydrazinium acetate, DMF, 40 °C, 4 h, 95%; (viii) Cl₃CCN, DBU, DCM, 0 °C, 30 min, 85%.

3.4 Synthesis of building block 3.5

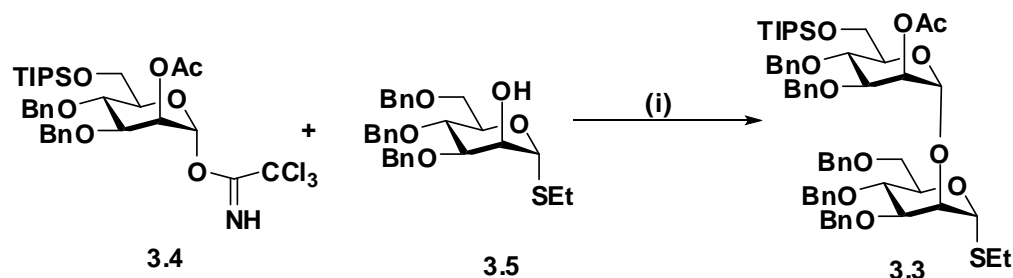
Building block **3.5** was synthesized using compound **3.8** as the starting material. The triol was first benzylated using benzyl bromide and sodium hydride to afford benzylated orthoester **3.13**, which when exposed to mercuric bromide and ethanethiol in acetonitrile at 60 °C which resulted in the regioselective opening of orthoester **3.13** to obtain thioglycoside **3.14** (Scheme 3.4).¹⁸⁴ Finally the Zémlen method was employed to deacetylate and obtain acceptor **3.5** (Scheme 3.4).



Scheme 3.4 (i) BnBr, NaH, DMF, 24 h, 88%; (ii) HgBr₂, EtSH, CH₃CN, 60 °C, 24 h, 78%; (iii) NaOMe, MeOH, 1 h, quant.

3.5 Synthesis of disaccharide 3.3

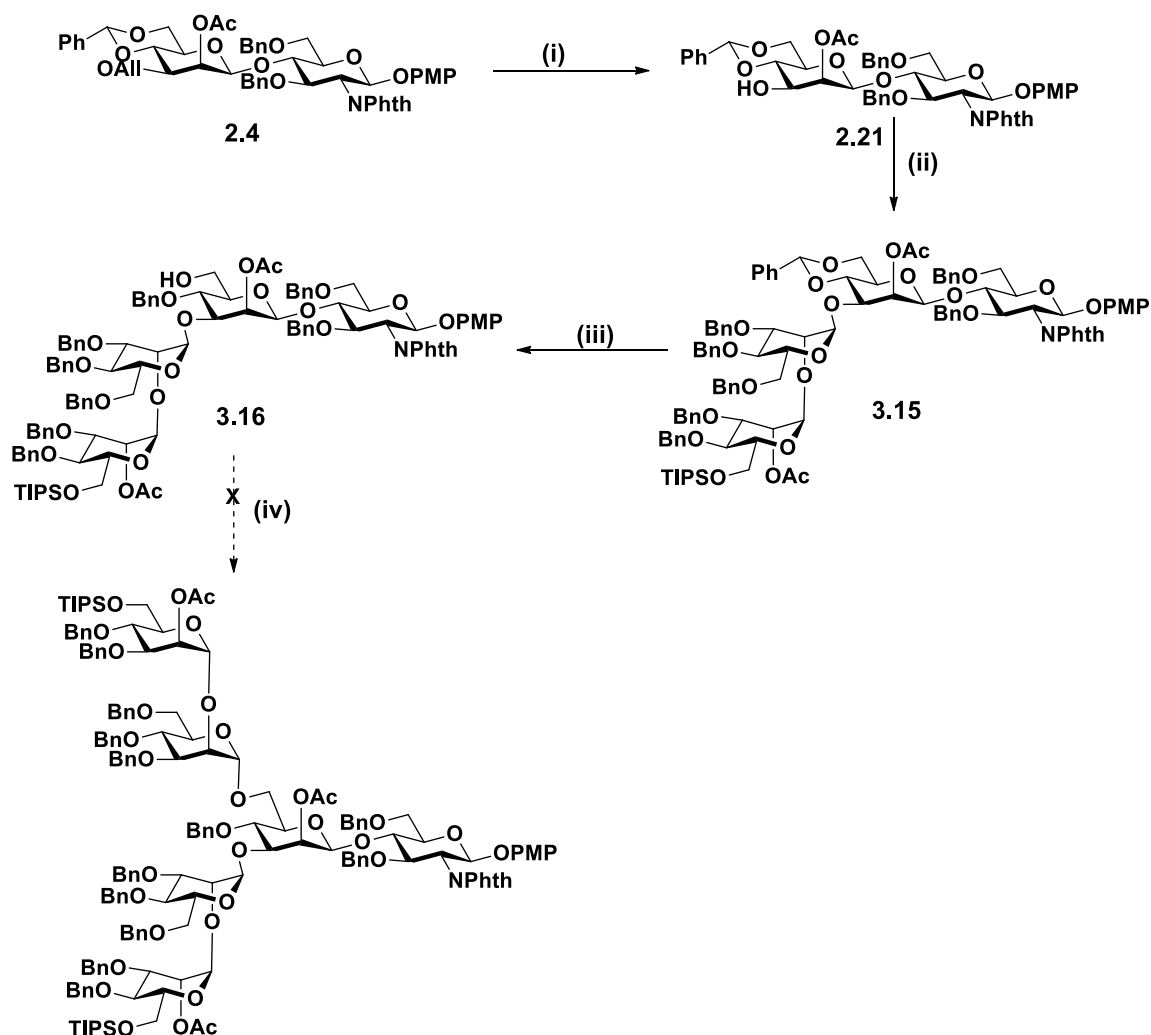
Glycosylation of the *manno* acceptor **3.5** with the imidate donor **3.4**, in presence of TMSOTf as the activator in DCM at 0 °C, resulted in formation of disaccharide **3.3** with complete α -selectivity, i.e. producing only the desired $\alpha(1\rightarrow2)$ linked disaccharide (Scheme 3.5).⁴⁹



Scheme 3.5 (i) TMSOTf, DCM, 0 °C, 30 min, 61%.

3.6 Hexasaccharide synthesis

With both disaccharides **2.4** and **3.3** in hand, attention turned to the synthesis of the hexasaccharide target **3.1**. The allyl ether of the disaccharide **2.4** was catalytically isomerized to the enol ether using (1,5-cyclooctadiene) bis(methyldiphenylphosphine) iridium(I) hexafluorophosphate activated with hydrogen,¹⁸⁰ followed by treatment with *N*-iodosuccinimide and water to afford the C-3 alcohol **2.21** (Scheme 3.6). Glycosylation of acceptor **2.21** with thioglycoside donor **3.3** was performed stereoselectively with methyl trifluoromethanesulfonate (MeOTf) and tri-*tert*-butylpyrimidine (*t*-TBP) to afford tetrasaccharide **3.15** in 55% yield (70% based on recovered acceptor). The 4,6-*O*-benzylidene of **3.15** underwent reductive ring opening to afford tetrasaccharide acceptor **3.16** in 83% yield in a highly regioselective manner following treatment with triethylsilane and dichlorophenylborane. However glycosylation of the tetrasaccharide acceptor **3.16** with the disaccharide donor **3.3** proved to be troublesome.



Scheme 3.6 (i) a) (1,5-cyclooctadiene) bis(methyldiphenylphosphine) iridium(I) hexafluorophosphate, THF, 14 h; b) NIS, H₂O, 18 h, 86% over 2 steps; (ii) MeOTf, 3Å mol. sieves, DCM, *t*-TBP, 0 °C to rt, 18 h 55%; (iii) Et₃SiH, PhBCl₂, 3Å mol. sieves, DCM, -78 °C, 50 min, 83%;

Initial attempts using methyl trifluoromethanesulfonate as activator in the presence tri-*tert*-butylpyrimidine did not result in the formation of any product. The starting materials were recovered and re-subjected to the same reaction conditions but unfortunately again no formation of product was observed. Alternative activators for thioglycosides were therefore assayed. Reactions using *N*-iodosuccinimide (NIS) and silver

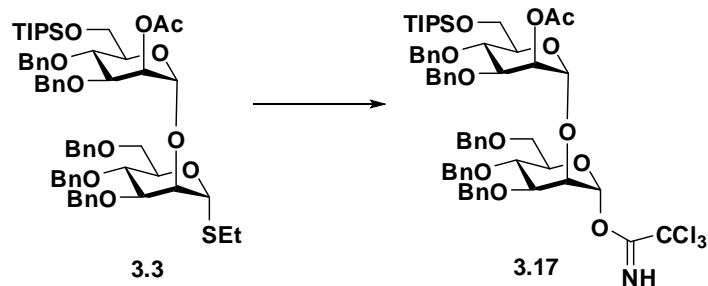
trifluoromethanesulfonate,¹⁸⁶ or NIS and trimethylsilyl trifluoromethanesulfonate were also unsuccessful, even after extended reaction times. Use of Crich's activation method^{58,186} using 1-benzene sulphonyl piperidine (BSP) and triflic anhydride resulted in the formation of significant quantities of decomposition products and no desired product could be isolated. Glycosylation using NIS and a catalytic amount of triflic acid did not lead to reaction of the starting material, whilst increasing the amount of triflic acid led to slow decomposition of the starting materials (Table 3.1).

Table 3.1 Summary of thioglycoside activation conditions used to attempt to synthesise the hexasaccharide

<i>Conditions</i>	<i>Outcome</i>
MeOTf, <i>t</i> -TBP, DCM, 0 °C, 18 h.	No reaction
NIS, AgOTf, DCM, 0 °C, 1 h.	Slow decomposition of starting material.
NIS, TMSOTf, -20 °C, 30 min.	Slow decomposition of starting material.
NIS, TfOH, -78 °C, 15 min.	Decomposition
BSP, Tf ₂ O, -78 °C, 10 min.	Decomposition

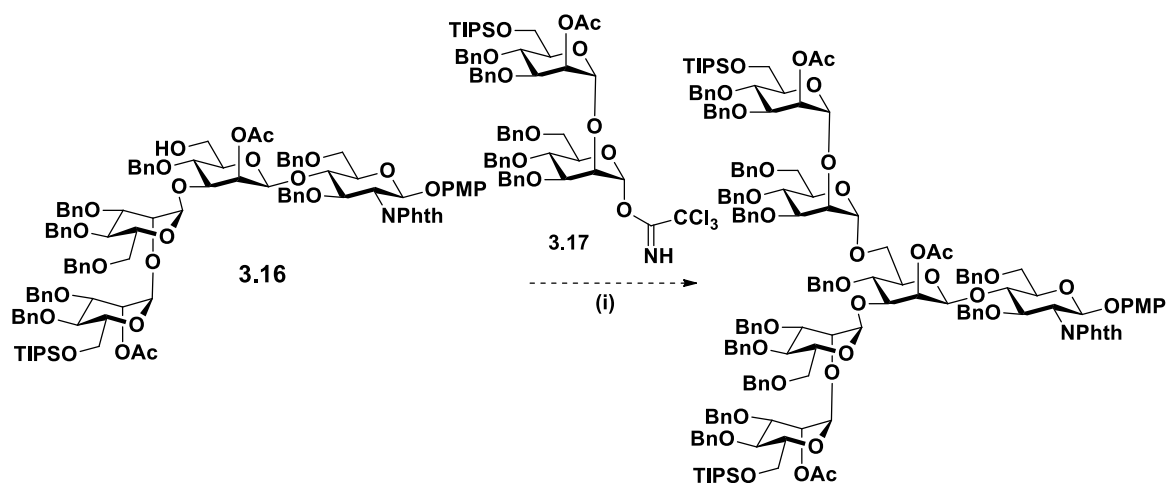
Since all efforts to glycosylate the thioglycoside were unsuccessful, it was decided to convert thioglycoside **3.3** to the trichloroacetimidate donor **3.17**, as imidates are known to be active glycosyl donors. This was done in 2 steps, firstly by converting the

thioglycoside **3.3** to the corresponding hemiacetal using NBS in wet acetone, and then treating the product with DBU and trichloroacetonitrile in DCM to afford **3.17** in 71% yield (Scheme 3.7).¹⁸⁷



Scheme 3.7 (i) Cl_3CCN , DBU, DCM, 0 °C, 30 min, 71%

Glycosylation of the tetrasaccharide acceptor **3.16** with trichloroacetimidate **3.17** using TMSOTf in DCM was attempted.^{188,189} Surprisingly, this also resulted in decomposition of the starting materials and no formation of product. Despite numerous attempted reactions we were not able to obtain the hexasaccharide; furthermore we were unable for the rationalise the failure of the reactions (Scheme 3.8).

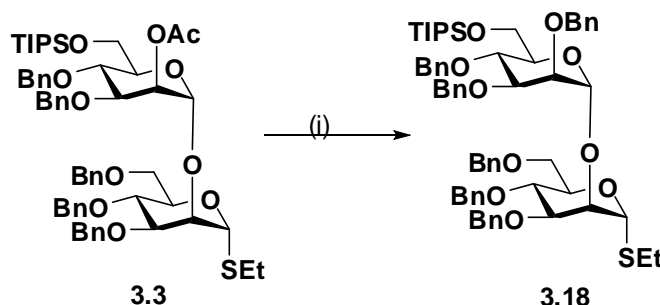


Scheme 3.8 (i) TMSOTf, DCM, 0 °C, 30 min, no reaction.

3.7 An alternate synthetic route to the hexasaccharide

In light of these failed reactions our attention turned towards a publication by Chen *et al.*⁴⁹ wherein the acetate at the C-2 position of the thioglycoside donor **3.3** had been converted to a benzyl group before glycosylation was performed a similar oligosaccharide.

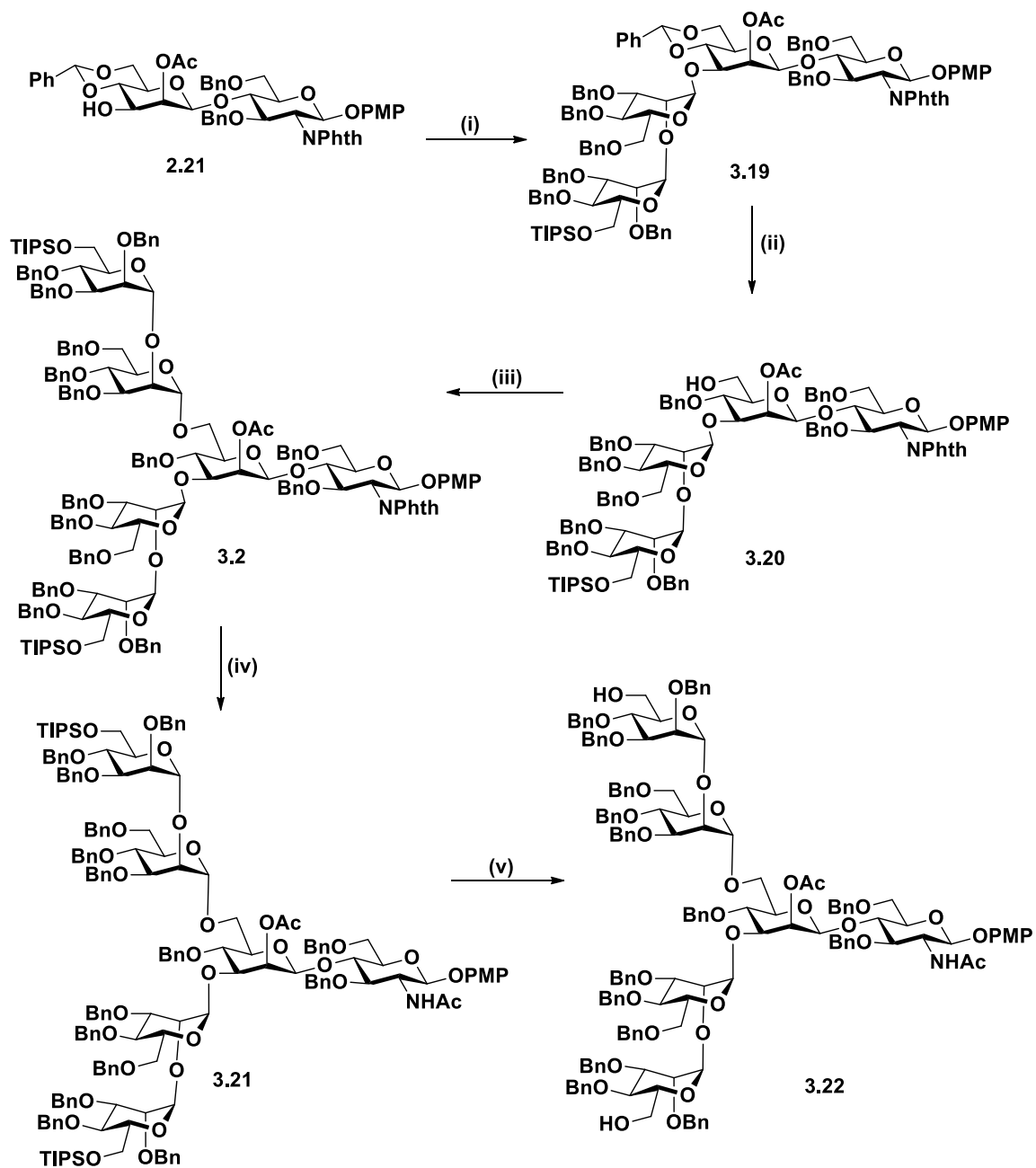
Disaccharide **3.3** was deacetylated using the Zemplen method. The C-2 hydroxyl group was then protected as a benzyl ether by treatment with benzyl bromide and sodium hydride in THF to obtain thioglycoside donor **3.18** in good yield (Scheme 3.9).⁴⁹



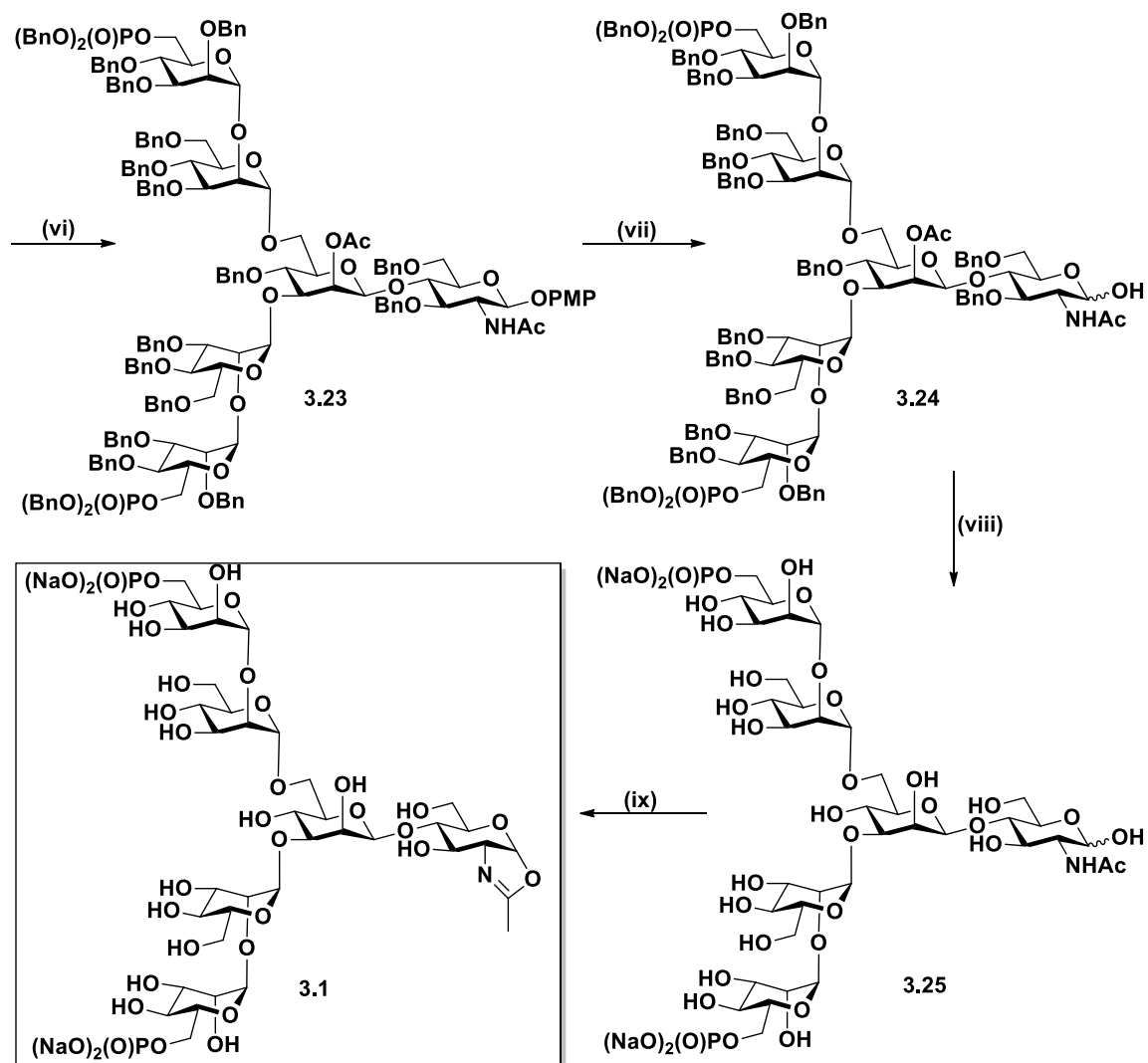
Scheme 3.9 (i) NaOMe, MeOH, 0 °C to rt, 18 h, 75%; ii) NaH, BnBr, DMF, 0 °C to rt, 18 h, 83%.

Glycosylation of acceptor **2.21** with donor **3.18**, using NIS and catalytic amount of TfOH as activator, afforded the tetrasaccharide **3.19** in 65% yield (Scheme 3.10a). Regioselective reductive benzylidene opening^{181,182} with Et₃SiH and PhBCl₂, gave the tetrasaccharide acceptor **3.20** in 78% yield. Pleasingly glycosylation of the tetrasaccharide acceptor **3.20** with thioglycoside **3.18** using NIS and triflic acid gave the desired hexasaccharide **3.2** in 72% yield. Hexasaccharide **3.2** was then treated with ethylene diamine and methanol at reflux to remove the phthalimide protecting group. The

crude amine was then acetylated to afford **3.21** in 71% yield over 2 steps. The TIPS groups were removed using $\text{BF}_3 \cdot \text{OEt}_2$ in 68% yield without any difficulty to obtain **3.22**. Bisphosphorylation of **3.22** using *N,N*-diisopropyl phosphoramidite in tetrazole (0.45 M solution in acetonitrile) followed by *m*-CPBA oxidation then gave **3.23** in 94% yield over two steps (Scheme 3.10b).⁵⁰ Oxidative cleavage of the anomeric *para*-methoxyphenyl glycoside was achieved using ceric ammonium nitrate (CAN) to obtain **3.24** in 80% yield.¹³⁰ Global de-protection was performed using Birch reduction to obtain the fully de-protected phosphorylated hexasaccharide **3.25** in 83% yield. The hexasaccharide was then converted to the desired oxazoline **3.1** by treatment with DMC and triethylamine using the method published by Shoda *et al* in 95% yield.¹⁵⁸



Scheme 3.10a (i) **3.18**, NIS, TfOH, 3 Å mol. sieves, DCM, -20 °C, 30 min, 65%; (ii) Et_3SiH , PhBCl_2 , 3 Å mol. sieves, DCM, -78 °C, 30 min, 78%; (iii) **3.18**, NIS, TfOH, 3 Å mol. sieves, DCM, -20 °C, 30 min, 72%; (iv) (a) $\text{NH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, MeOH, reflux, 18 h; (b) Ac_2O , py, 18 h, 71%; (v) $\text{BF}_3 \cdot \text{OEt}_2$, DCM, 0 °C, 1 h, 68%.



Scheme 3.10b (vi) $(\text{BnO})_2\text{PNiPr}_2$, tetrazole, DCM, rt, 18 h then m-CPBA, -78°C , 2 h, 94%; (vii) CAN, MeCN, H_2O , rt, 1 h, 80%; (viii) Na, $\text{NH}_3(\text{l})$, -33°C , 83%; (ix) DMC, Et_3N , H_2O , rt, 30 min, 95%.

3.8 Conclusion

Target hexasaccharide **3.1** was successfully synthesized in 6% overall yield in 40 steps based on the longest linear sequence. The main obstacle encountered was the

glycosylation reaction required to obtain the hexasaccharide **3.2**. Different glycosylation conditions were tried, and, following a protecting group swap it was found that NIS and triflic acid activation of a thioglycoside donor gave a successful result. Global deprotection was performed without decomposition of the hexasaccharide. Furthermore, it was found that oxazoline formation was quite straightforward using Shoda's method. It is clear that using this approach, various other biologically important phosphorylated oligosaccharides could be readily synthesized.

Chapter 4

Enzyme- catalyzed glycosylation reactions

4.1 Introduction

In order to synthesize structurally defined oligosaccharides and glycoconjugates, a variety of elegant chemical and enzymatic methods have been developed.^{39,190-192} Notably, in contrast to conventional chemical synthetic methods that usually require tedious protection and de-protection manipulations in order to achieve regio- and stereo-selectivity, enzymatic glycosylation usually provides perfect control of anomeric configuration and high regioselectivity without the need for any protecting groups. Both glycosyltransferases and glycosidases have been extensively studied for synthetic purposes.^{193,194}

4.2 Glycosyltransferases

Glycosyltransferases synthesize oligosaccharides, polysaccharides and glycoconjugates by transferring activated sugar-phosphate substrates (e.g. UDP-galactose) to acceptors. Generally they act in a stepwise manner and are highly specific with respect to their substrates.^{102-104,106,107} Glycosyltransferases that use sugar nucleoside phosphates as the donors are known as Leloir enzymes, named after Luis F. Leloir, the scientist who discovered the first sugar nucleotide and received the 1970 Nobel Prize in Chemistry for his work on carbohydrate metabolism. Glycosyltransferases that utilize non-nucleoside donors, e.g. sugar phosphates, are termed non-Leloir glycosyltransferases.

Glycosyltransferases have proven to be extremely useful enzymes for building complex carbohydrates,^{102,104,105} but there are several drawbacks. Glycosyltransferases usually require complex sugar nucleoside phosphates as the donor, and have very stringent

substrate specificity. There is an additional complication in cases where the nucleoside diphosphate by-products are inhibitors of the enzymes.¹⁹⁵

4.3 Glycosidases

Glycosidases play many important and varied roles in biological systems,¹⁹⁶ ranging from processes such as degrading polysaccharide food sources to manipulation of glycoconjugate structures on the surfaces of proteins and cells. Glycosidases are of two types; 1) exoglycosidases, which cleave the glycosidic linkage to release the terminal monosaccharide residue of an oligosaccharide and, 2) endoglycosidases, which have the ability to cleave internal glycosidic bonds in an oligosaccharide chain or glycoconjugate. Under controlled conditions, glycosidases can be used to synthesize glycosidic bonds rather than to cleave them.¹⁹⁵ As such, they have been extensively utilised as biocatalysts for oligosaccharide synthesis.¹⁹⁷⁻²⁰⁰

4.3.1 Mechanism of glycosidases: retaining and inverting mechanisms

There are over 1000 different glycosyl hydrolases which are currently split into 133 families.²⁰¹ These are sub-divided in two main classes: retaining hydrolases and inverting hydrolases, a classification which is based on the stereochemical outcome of the hydrolysis reaction. The active site for both classes of enzymes usually contains two conserved carboxylic acid residues (Figure 4.1). The distance between the two carboxylic acids is usually indicator of whether a glycoside is mechanistically retaining or inverting. This distance is typically 5.5 Å for retaining, whereas it is 10 Å (+/- 2 Å) for inverting glycosidases.^{202,203}

Hydrolysis of a glycoside with net inversion of anomeric configuration is generally achieved *via* a one step, single-displacement mechanism involving an oxocarbenium -like transition state. Both carboxylic acids are positioned such that one carboxylic acid acts as a general acid to protonate the glycosidic oxygen and promote cleavage of the glycosidic bond. At the same time the second carboxylic acid acts as a general base to promote attack of the nucleophilic water molecule (Figure 4.1).

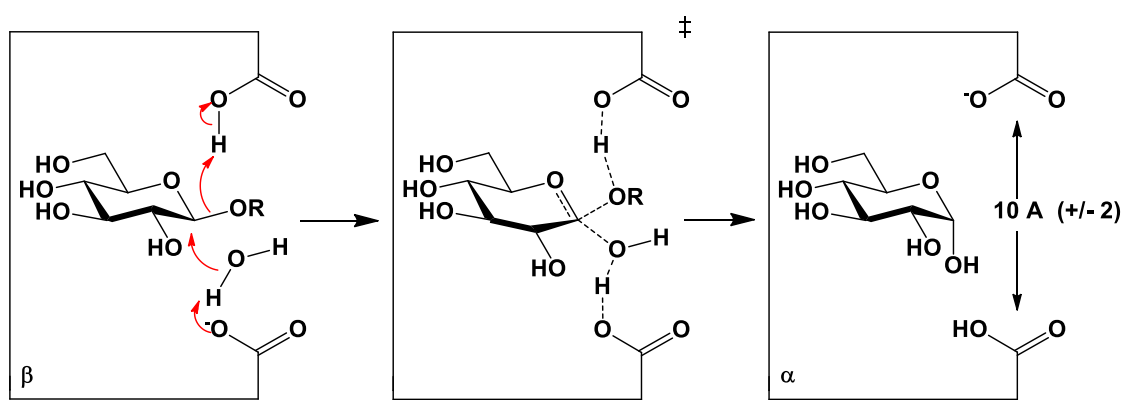


Figure 4.1 Mechanism of inverting glycosidases

Hydrolysis with net retention of configuration is most commonly achieved *via* a two-step double-displacement mechanism involving a covalent glycosyl-enzyme intermediate that is subsequently hydrolysed (Figure 4.2). One carboxylic acid acts as a general acid to promote cleavage of the glycosidic bond in the same way as for inverting glycosidases. In this mechanism, however, the second carboxylic acid residue acts as a nucleophile to displace the glycosidic oxygen and form a covalent glycosyl-enzyme intermediate.²⁰⁴ The first acid residue then acts as a general base to promote nucleophilic attack by a water molecule onto this glycosyl-enzyme intermediate.

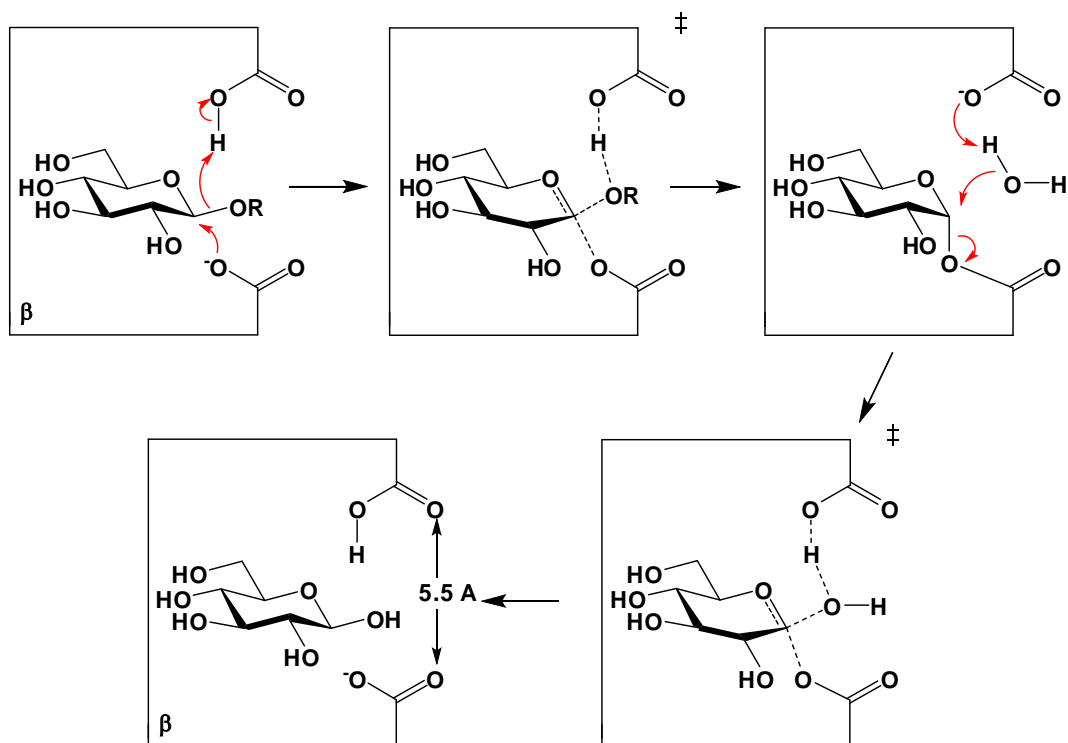


Figure 4.2 Mechanism of retaining glycosidases

4.3.2 Use of glycosidases for synthesis: thermodynamic vs kinetic approaches

In the thermodynamic approach (reverse hydrolysis), synthesis can be favored over hydrolysis by using large excess of substrate,¹⁹⁵ organic solvents, high salt concentrations,²⁰⁵ or high temperatures¹⁹³. Unfortunately, the thermodynamic approach usually results in low yields and is generally not regioselective, and so often results in the formation of mixtures of products. Improved yields can often be achieved under conditions of kinetic control (Figure 4.3).^{141,195} Thus, in order to obtain a good yield, the rate of hydrolysis must be slower than the rate of transglycosylation; one method of favouring transglycosylation over hydrolysis is to use high acceptor concentrations.

Furthermore, in order to achieve kinetic control the transglycosylation product must itself react more slowly than the activated donor used. Anomeric fluorides and *p*-nitrophenyl glycosides are two of the activated glycosides that are commonly used donors for glycosidase mediated transglycosylation reactions.

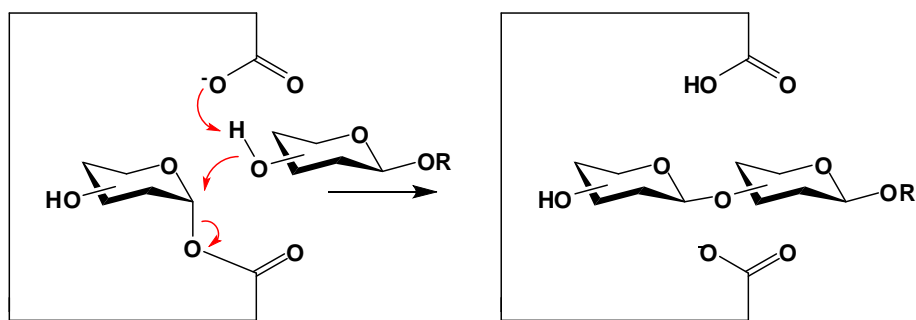


Figure 4.3: Kinetic transglycosylation by interception of the covalent enzyme-bound intermediate using a retaining glycosidase

4.4 Endoglycosidases

Endoglycosidases cleave internal glycosidic bonds in an oligosaccharide chain or glycoconjugate. Some endoglycosidases, like chitinases and endo- β -*N*-acetylglucosaminidases, can also catalyze transglycosylation reactions^{117,206} where they transfer intact oligosaccharide units.

4.4.1 Chitinases

Chitinases, which range in size from 20 kDa to about 90 kDa,²⁰⁷ catalyze the hydrolysis of chitin, a $\beta(1\rightarrow4)$ GlcNAc polysaccharide which is found as a structural components of many organisms. There are five known classes of chitinase which are further classified into two glycosyl hydrolase families.²⁰⁸ Family 18 chitinases are found in wide range of

organisms, including plants, bacteria, fungi and animals, whereas family 19 chitinases are found mainly in plants.^{208,209}

The hydrolytic mechanism is different for the two hydrolase families. Family 19 use an inverting mechanism similar to that described before, going *via* a single displacement mechanism.²⁰⁸ Family 18 uses a retaining mechanism *via* anchimeric assistance involving an oxazolinium intermediate (Figure 4.4).²⁰⁹⁻²¹¹ The oxygen atom of the glycosidic bond in first protonated by a carboxylic acid in the active site (general acid) and the oxazolinium ion intermediate is then formed by nucleophilic attack by the neighbouring acetamido group at the anomeric centre. The oxazolinium intermediate is then attacked by the incoming nucleophile; water in the case of a hydrolysis reaction and a sugar acceptor in the case of transglycosylation.

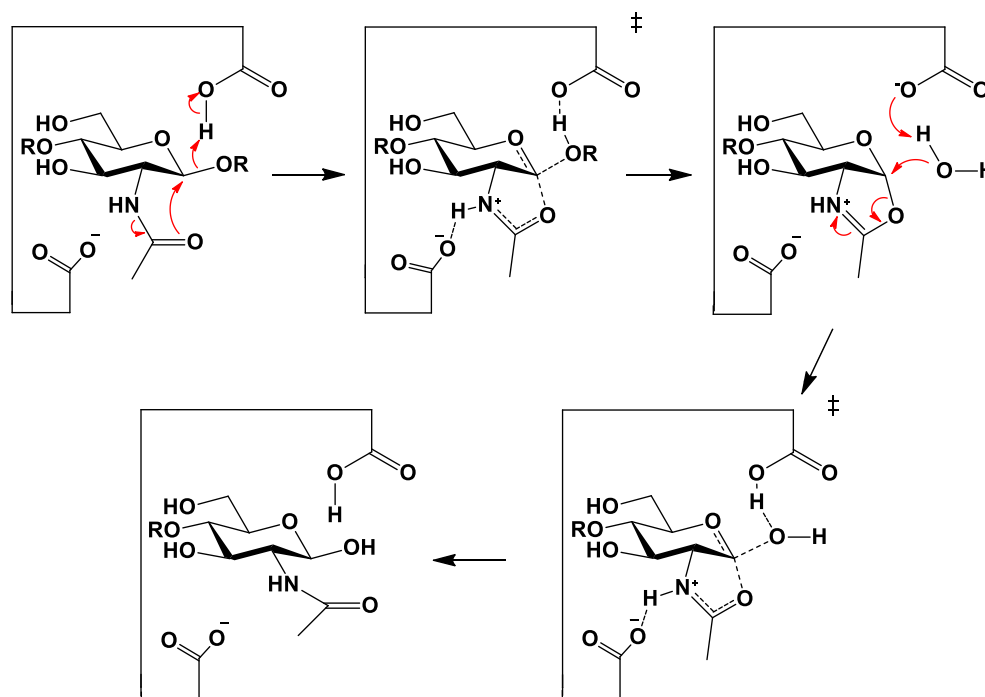
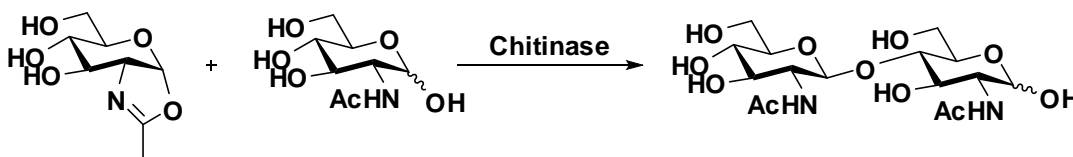


Figure 4.4 Mechanism of glycosyl hydrolase family 18 enzymes

Various acceptors have been successfully glycosylated with GlcNAc using chitinases.^{205,212} The donors used include glycosides, phenylglycoside derivatives, and glycosyl fluorides. However, hydrolysis also takes place because the product of the reaction is a substrate for the enzyme.

In 1996, Kobayashi *et al.* demonstrated the use of chitobiose oxazoline as a transition state mimic for glycosylation catalyzed by chitinase.²¹³ At pH 7.8, the glycosylation reaction gave 40-50% yield of artificial chitin after six hours. When the glycosylation was performed at pH 10.6, a quantitative yield of product was obtained after 50 hours. Interestingly no hydrolysis took place because the pH required for enzymatic hydrolysis is pH 7.8. Later they also used this methodology to synthesise *N, N'*-Diacetylchitobiose (Scheme 4.1), the smallest repeating unit of chitin, by combining a sugar oxazoline derivative as a new glycosyl donor and *N*-acetylglucosamine as a glycosyl acceptor for chitinase.²¹⁴



Scheme 4.1 Synthesis of *N, N'*-Diacetylchitobiose

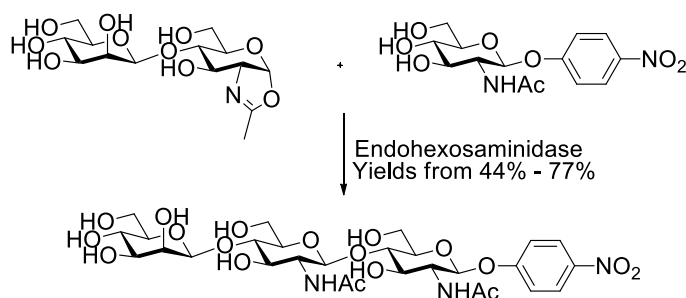
4.4.2 Endo- β -*N*-acetylglucosaminidases (ENGases)²¹⁵

Endo- β -*N*-acetylglucosaminidases (ENGases)²⁰¹ are a group of endoglycoside hydrolases which catalyze the hydrolysis of the glycosidic bond between the two proximal *N*-acetylglucosamines [GlcNAc β (1 \rightarrow 4)GlcNAc] (known as the chitobiose core),²¹⁶ linked

to asparagine in *N*-linked glycans, leaving one *N*-acetylglucosamine residue attached to asparagine, whilst the other becomes the reducing end of the detached *N*-glycan.²¹⁷ Endohexosaminidases differ significantly from the chitinases in their specificity. Whereas chitinases can only cleave chitobiose, endohexosaminidases can cleave high mannose, hybrid, and even complex *N*-glycans depending on the individual specificity of particular enzymes.

Members of this group of enzymes are found in both families 18 (GH18) and 85 (GH85) of the glycoside hydrolases.²⁰¹ Four endo- β -*N*-acetylglucosaminidases that are members of family GH85, which are produced by both eukaryotes and prokaryotes, are Endo-M from *Mucor hiemalis*,¹¹¹ Endo-CE from *Caenorhabditis. elegans*,²¹⁸ Endo-A from *Arthrobacter protophormiae*²¹⁹ and Endo-D from *Streptococcus pneumoniae*.²²⁰ The family GH18 enzymes are related to chitin-degrading chitinases and contained, until recently, only endo- β -*N*-acetylglucosaminidases solely expressed by prokaryotes including Endo-H from *Streptomyces plicatus*,²²¹ Endo-F1, F2, F3 from *Elizabethkingia meningoseptica*²²² and Endo-S from *Streptococcus pyogenes*.²²³ Both family GH18 and family GH85 ENGases are thought to use the same retaining mechanism, but this is not confirmed.²²⁰

In 2001, Shoda and co-workers found that oxazolines could be used as transition state mimics for some ENGases. Both Endo A and Endo M were capable of performing a glycosylation reaction using a disaccharide oxazoline donor substrate with *p*-nitrophenyl-*N*-acetylglucosamine as an acceptor (Scheme 4.2).¹²⁶



Scheme 4.2 ENGase transglycosylation with oxazoline donors

This report further stimulated interest in the potential synthetic applications of these enzymes by a number of research groups. The groups of Wang and Fairbanks have investigated various aspects of this reaction (see 1.8.1 and 1.8.2).^{121,127,128,136,140,156} Mutants of Endo A and Endo M have also been used for this reaction to further improve the synthetic efficiency of the ENGase catalysed reactions.^{129,135} The donor and acceptor requirements for the enzymes have also been studied.^{127,128,136,140} Synthetic application of these enzymes has the potential to provide a generalized and high-yielding method for the synthesis of homogeneous glycoproteins which bear *N*-glycan oligosaccharides of desired structure.

4.5 Glycosynthases

4.5.1 Introduction

Glycosynthases are a type of engineered mutant glycosidases that can irreversibly catalyze the formation of a glycosidic bond from a glycosyl donor and an acceptor alcohol.²²⁴ In 1998, various research groups investigated mutagenesis of the catalytic active site residues and their use for the synthesis of oligosaccharides.^{225,226} Studies on β -

glycosidases showed that changing the nucleophilic carboxylic acid residue in the active site of a retaining glycosidase to a non-nucleophilic amino acid (such as alanine) removed hydrolytic activity. In the absence of nucleophilic activity, the covalent glycosyl-enzyme intermediate could not be formed and so hydrolysis could not take place. However Planas and Withers found that activated α -glucosyl fluorides were still substrates for these enzymes. Furthermore the enzymes were capable of catalyzing the transfer of these glucose units to acceptor sugars, to give products with net inversion of configuration, but which were not hydrolyzed by the enzymes. Withers termed these enzymes ‘glycosynthases’ (Figure 4.5).^{225,227}

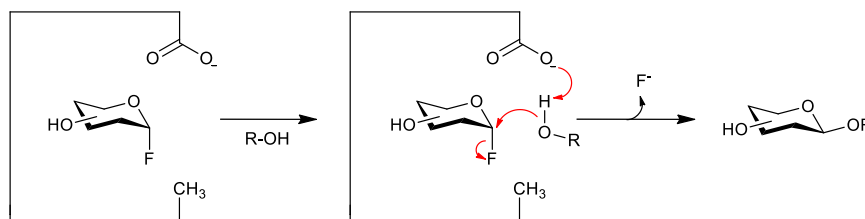


Figure 4.5: Glycosynthases – general mechanism

More recently, glycosynthases derived from inverting glycosidases have been developed. However, there is no ‘general strategy’ for their engineering.²²⁸⁻²³⁰ Glycosynthases have also been employed for the synthesis of xylo oligosaccharides,²³¹⁻²³³ and xyloglucans.²³⁴

4.5.2 Classification

Glycosynthases, like glycosidases, can be divided in two classes; endo- or exo-glycosynthases, depending on the specificity of the glycosidase from which they were engineered.²³⁵ Endoglycosynthases catalyze the formation of specific glycosidic linkages

and they are highly regiospecific. The active site of endoglycosynthases can accommodate large oligosaccharide acceptors and large donor substrates, and can generate products with high degrees of polymerization. Exoglycosynthases have a more relaxed substrate specificity and regioselectivity, and their products are mostly short-chain oligosaccharides. Further details about glycosynthases have been discussed in Chapter 1.

4.6 Objectives

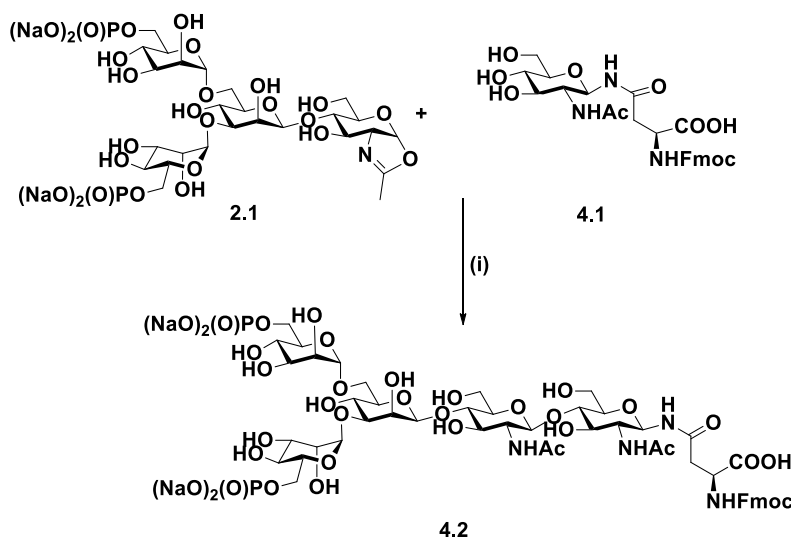
- To investigate whether ENGases can process phosphorylated *N*-glycan oxazoline as donors and allow the production of the first synthetic *N*-linked glycoproteins that contains M6P residues in which the glycans are linked to the peptide by native linkages.
- To apply this method to full-length *N*-glycan structures to obtain homogeneous phosphorylated *N*-glycan structures that are the natural ligands for M6P receptors.
- To apply this method to produce recombinant lysosomal hydrolases, which may be used for the treatment of lysosomal storage diseases by Enzyme Replacement Therapy (ERT).

4.7 ENGase catalysed glycosylation of amino acid acceptor using tetrasaccharide oxazoline 2.1

ENGase-catalysed glycosylations were carried out using the phosphorylated tetrasaccharide oxazoline **2.1** as the donor and glycosyl amino acid **4.1** as the acceptor.

This acceptor has been previously used to investigate a variety of other ENGase catalysed glycosylations using non-phosphorylated N-glycan oxazolines as donors.^{131,138,236} The acceptor **4.1** bears a chromophore to facilitate HPLC analysis of the reaction efficiency *via* UV detection.

It was found that WT Endo A, which does not normally operate hydrolytically on *N*-glycans that bear a negative charge, was able to catalyze transfer of the oxazoline donor **2.1** to the acceptor **4.1**. Reaction of **2.1** and **4.1** and WT Endo A (1 μ g) was conducted using three equivalents of the donor and one equivalent of the acceptor in a 100 mM sodium phosphate buffer at *pH* 6.5 at 37 °C and gave product **4.2** in 40% yield after 2 hours (Scheme 4.3, Figure 4.8). The further addition of six equivalents of donor **2.1** increased the yield to 73%. A full time course study of this particular reaction was undertaken (Figure 4.7), which revealed that, although the reaction product **4.2** was a hydrolytic substrate for WT Endo A, hydrolysis occurred only very slowly



Scheme 4.3 (i) ENGase, phosphate buffer *pH* 6.5, H_2O , 37 °C, 2 h.

Endo M has a broader hydrolytic capability than Endo A, as it has the ability to hydrolyse complex biantennary *N*-glycans as well as high mannose structures.^{127,130} However investigation under identical conditions that were used for Endo A revealed that Endo M was unable to catalyze the synthesis of **4.2**. Glycosylation with the use of a commercially available glycosynthase mutant of Endo M, N175Q, was also performed. In this mutant, Asn175 which deprotonates the oxazolinium intermediate was mutated to glutamine. This effectively stops the hydrolytic activity of the enzyme, but it is still be able to perform the glycosylation reaction using an oxazoline as donor. However, reaction with the N175Q mutant did not result in glycosylation of **2.1** with **4.1** and only resulted in the hydrolysis of oxazoline **2.1**.

Surprisingly, Endo D, which has considerably more specific structural requirements with respect to the *N*-glycans it will hydrolyse as compared to both Endo A and Endo M,²³⁷ was able to catalyse the production of **4.2**, albeit in a very low yield (3%).

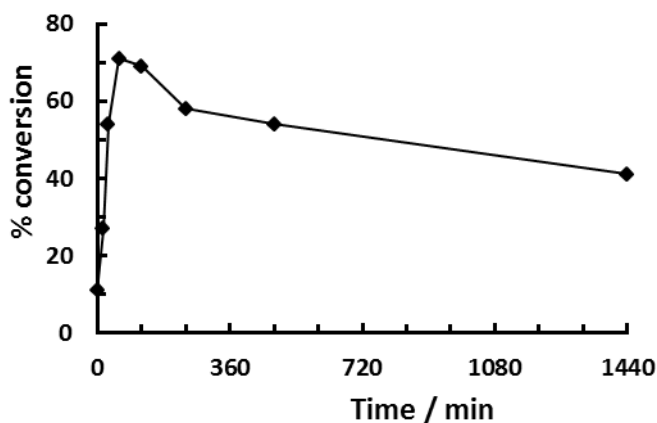


Figure 4.7 Time course study of glycosylation of **4.1** with **2.1** catalyzed by Endo A

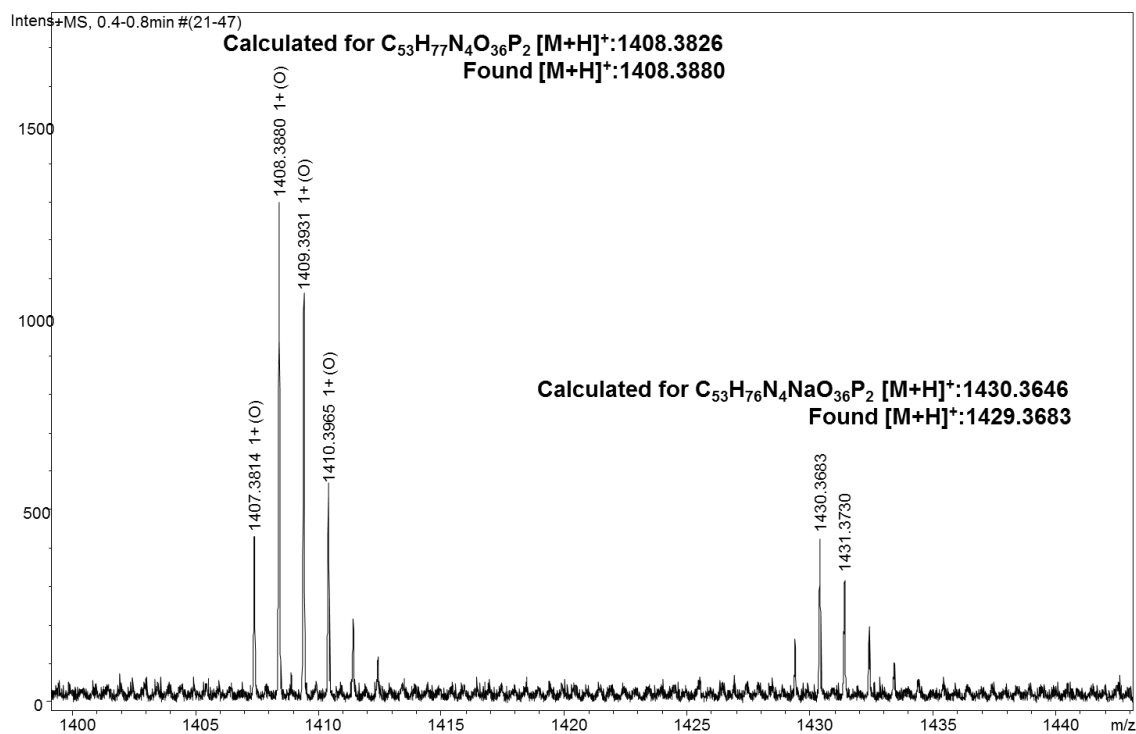
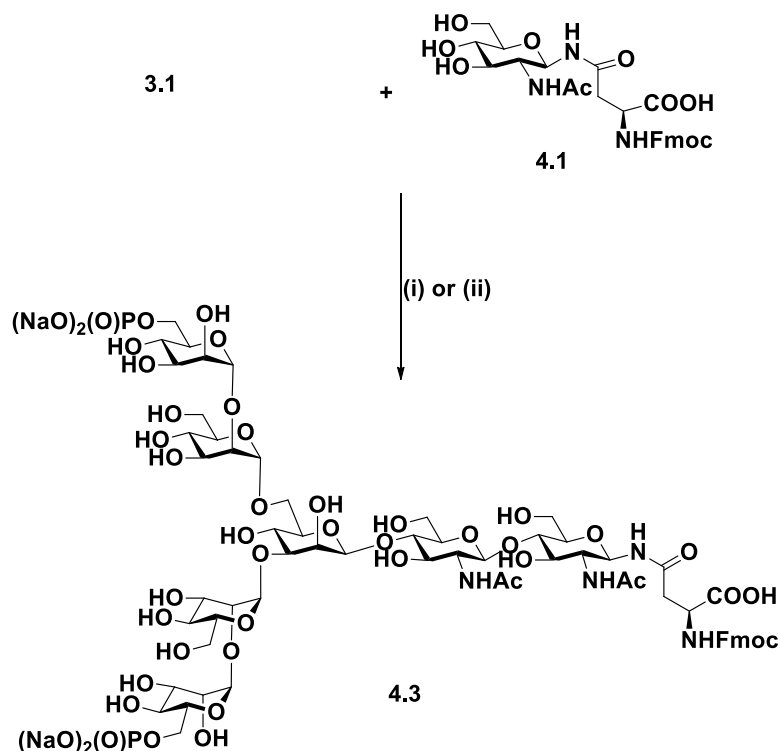


Figure 4.8 HRMS data for glycosyl amino acid 4.2

These results indicate that oxazoline **2.1** is a good donor substrate for Endo A but not for Endo M or its N175Q mutant. However the use of WT Endo A allowed the first successful synthesis of a phosphorylated *N*-glycan glycosyl amino acid using a chemo-enzymatic approach. Furthermore, these results also showed that phosphorylated *N*-glycan oligosaccharides can be processed by ENGases.

4.8 ENGase catalysed glycosylation of amino acid using hexasaccharide donor 3.1

As the synthesis of **4.2** had been successfully achieved, the next aim was to use the same approach to couple to a GlcNAc-Asn amino acid to a larger phosphorylated oligosaccharide oxazoline donor, the structure of which better corresponded to a full length *N*-glycan structure bearing M6P residues, and which was also deemed optimal for binding the M6PRs. Besides providing glycoconjugates with optimal M6PR binding affinities, this would also help to develop a better understanding of the relationship between the synthetic and hydrolytic efficiencies of the ENGases and the chain length of the phosphorylated *N*-glycan oligosaccharide. ENGase catalysed glycosylations were therefore carried out using the phosphorylated hexasaccharide oxazoline **3.1** as the donor and glycosyl amino acid **4.1** as the acceptor, under the same standard reaction conditions (Scheme 4.4).



Scheme 4.4 (i) ENGase, phosphate buffer *pH* 6.5, H₂O, 37 °C, 2 h.

WT Endo A was able to catalyze the transfer of the oxazoline donor **3.1** to acceptor **4.1**, and the product **4.3** was produced, albeit in low yield (12%) after two hours. The use of extended reaction times did not improve the amount of product formed, and hydrolysis of the oxazoline **3.1** took place. A full time course study of this reaction (Figure 4.9) revealed that, although the reaction product **4.3** was a hydrolytic substrate for WT Endo A, hydrolysis occurred only very slowly. When the glycosylation reaction was performed using WT Endo M, the Endo M mutant N175Q, both were able to catalyze the reaction, and **4.3** was obtained in 6% and 8% yields respectively after four hours (Figure 4.10). The remaining oxazoline **3.1** was hydrolysed over this time. The ability of Endo M to catalyse the synthetic reaction using the hexasaccharide donor **3.1** as a substrate, which

contrasts with the failure of the analogous reaction using the tetrasaccharide **2.1**, is a clear indicator of the effect of increased chain length. In the case of **3.1** the phosphate moieties are further from the reaction site, and so their presence has a smaller influence on the enzyme activity.

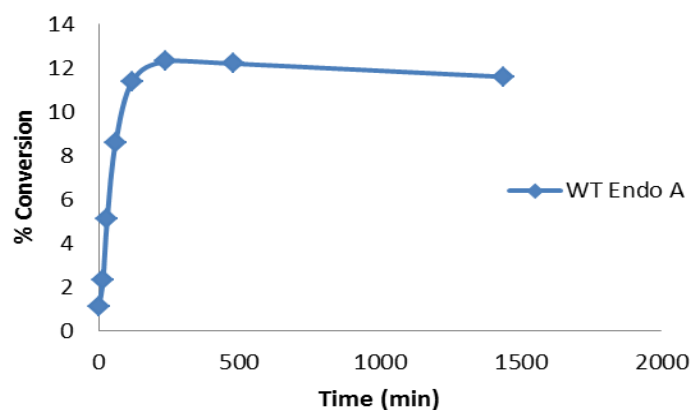


Figure 4.9 Time course study of glycosylation of **4.1** with **3.1** catalyzed by Endo A

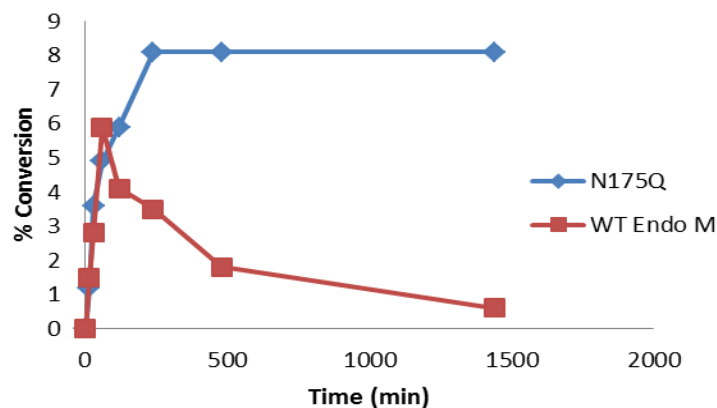


Figure 4.10 Time course study of glycosylation of **4.1** with **3.1** catalyzed by Endo M and N175Q

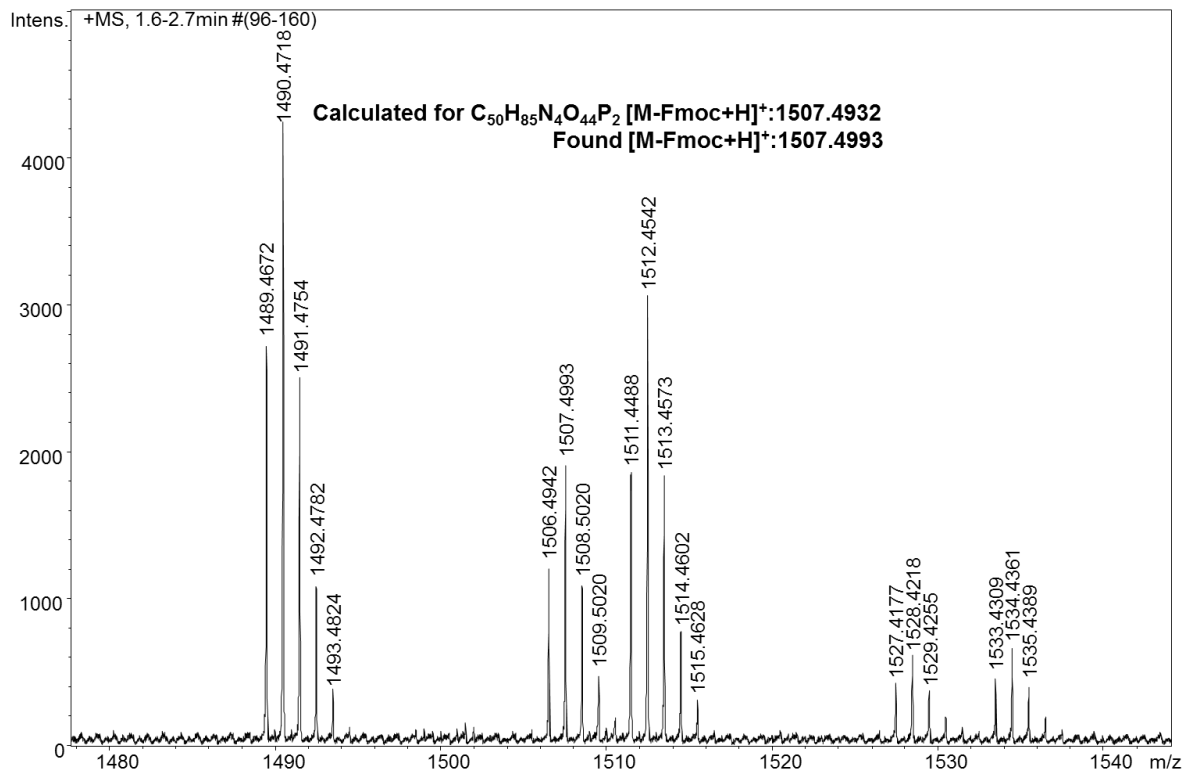


Figure 4.11 HRMS data for glycosyl amino acid 4.3

4.9 Glycosylation of 2.1 and 3.1 with dRNase B

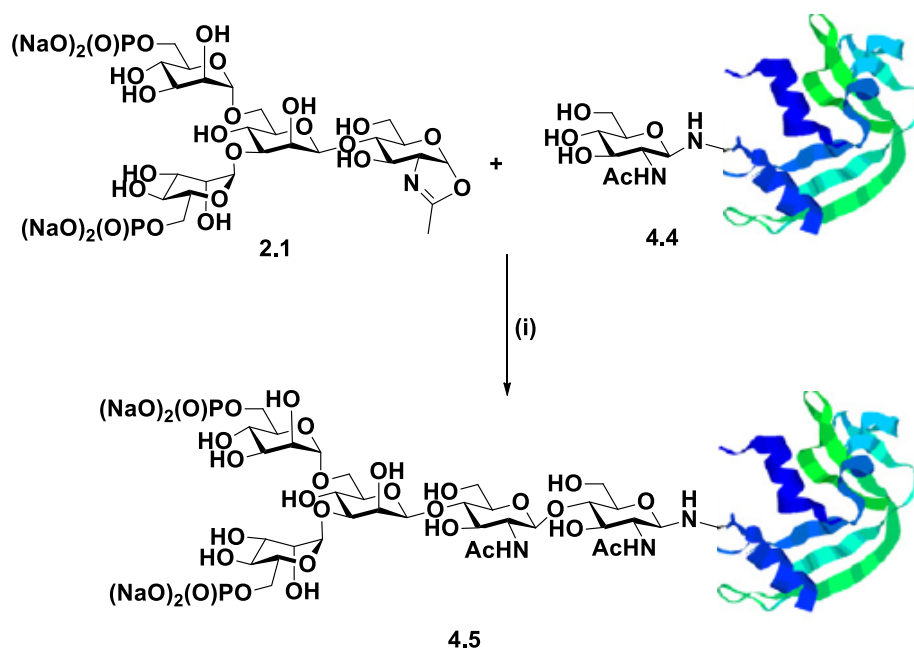
With the synthesis of phosphorylated glycosyl-amino acids successfully achieved, attention turned to the potential application of this approach for the production of phosphorylated glycoproteins. RNase B, which is commercially available as a mixture of high mannose glycoforms, was trimmed back by treatment with Endo H to produce dRNase B, a glycoprotein possessing a single GlcNAc residue at the sole glycosylation site. Partially glycosylated ribonuclease B (dRNase B) has previously been used as a model acceptor for enzyme catalyzed glycosylations using Endo A.^{134,137}

Firstly, WT Endo A mediated glycosylation of dRNase B **4.4** was performed using 3 equivalents of the tetrasaccharide donor **2.1** in a 100 mM sodium phosphate buffer at pH 6.5 at 37 °C, to obtain phosphorylated glycoprotein **4.5** (Scheme 4.5). Product formation was confirmed by HRMS (Figure 4.12). As no peak separation could be seen by using reverse phase HPLC, the reaction was monitored using SDS-PAGE (see Appendix); where the yield appeared to be approximately 20%. Different MeCN/H₂O gradient systems were tried in order to separate the starting material and product peaks using C-18, C-8 and C-5 reverse phase columns, but unfortunately all attempts were unsuccessful and purification of the product **4.5** could not be achieved.

Next, we tried to use a Concanavalin A column (Con A) to purify the product. Con A is known to bind to high-mannose type glycans, *N*-linked hybrid type glycans, and some *N*-linked biantennary complex type glycans.^{238,239} However, when the mixture of deglycosylated and glycosylated proteins were passed through a Con A column, no distinct peaks were seen. While carrying out this work, Wang and co-workers published their work on the chemoenzymatic synthesis and lectin recognition of a fluorinated glycoprotein.²³⁶ They reported that the difluoro-Man₃GlcNAc₂ glycoform (Scheme 4.6) showed only a very weak affinity for Con A. These results indicate that the C-6 hydroxyl group is important for the binding of the mannose residues to Con A and the presence of fluorine or a phosphate group likely blocks Con A binding.

Finally, hydroxyapatite (HAP) affinity chromatography was used to try and purify the glycoprotein. HAP is a crystalline form of calcium phosphate with the chemical formula, Ca₁₀(PO₄)₆(OH)₂. Several studies have demonstrated that phosphorylated proteins bind

more strongly to HAP matrices than their unphosphorylated counterparts. Therefore, it was expected that phosphorylated glycoproteins should selectively bind to HAP. However, on passing the protein mixture through a HAP column, a broad peak was seen, which, when analysed by MS, showed both deglycosylated and glycosylated proteins. Purification might be achieved, by changing the solvents and gradients and this remains one of the main objectives for the future.



Scheme 4.5 (i) ENGase, phosphate buffer pH 6.5, H₂O, 37 °C, 2 h.

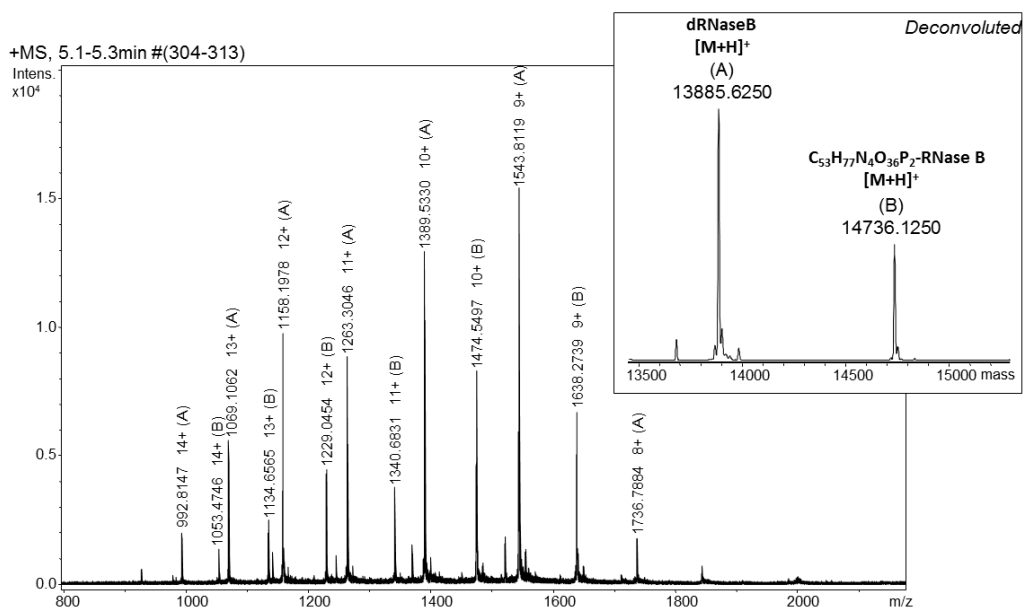
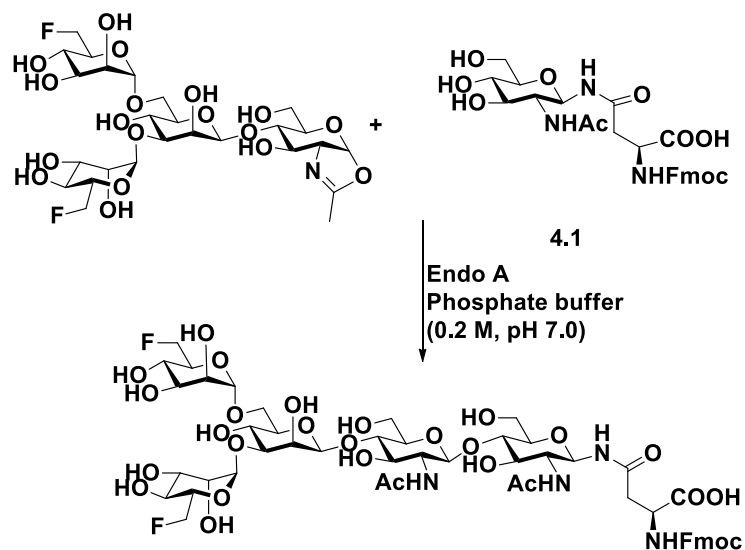


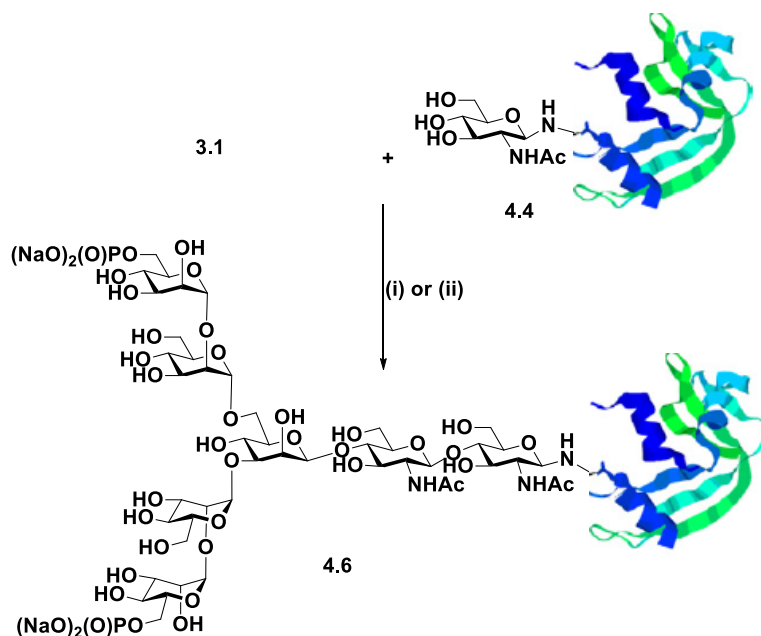
Figure 4.12 HRMS data for 4.5

Scheme 4.6 difluoro-Man₃GlcNAc₂ glycoform synthesized by Wang *et al.*²³⁶

Next WT Endo A mediated glycosylation of dRNase B **4.4** with hexasaccharide oxazoline was undertaken under standard reaction conditions: 6 equivalents of the

hexasaccharide donor **3.1** in a 100 mM sodium phosphate buffer at pH 6.5 at 37 °C the phosphorylated glycoprotein **4.6** was obtained (Scheme 4.7). The formation of the product was confirmed by HRMS. It was expected that the purification of the phosphorylated glycoprotein may be achieved easily, but, to our surprise again both the dRNase and the glycosylated protein merged together as a broad peak using HPLC and proved difficult to purify.

Nevertheless, these investigations indicated the potential of WT Endo A to catalyze the synthesis of glycoforms of glycoproteins which bear M6P-terminated *N*-glycans. With these results in hand, attention turned to the application of this approach to produce a phosphorylated version of a recombinant lysosomal hydrolase that has current therapeutic application. In principle the production of such glycoproteins, which bear phosphorylated *N*-glycans with optimal binding affinities for the M6PRs, should improve the efficacy of treatment of several lysosomal storage diseases by enzyme replacement therapy.



Scheme 4.7 (i) ENGase, phosphate buffer pH 6.5, H_2O , 37 °C, 1 h.

4.10 Glycosylation of de-glycosylated fabrazyme with 2.1 and 3.1

Fabrazyme was sourced from the laboratory of Prof Fran Platt (Department of Pharmacology, University of Oxford). Fabrazyme contains three *N*-glycosylation sites: N108, N161 and N184. Complex oligosaccharides are known to be present at Asn 108, whereas oligomannose (predominantly phosphorylated) structures were present at Asn 161 and 184.

Fabrazyme was deglycosylated with WT Endo A in sodium phosphate buffer (100 mM, pH 6.5) at 37 °C for 10 h (Scheme 4.7) in order to cleave the oligomannoses present at sites 161 and 184 without cleaving the complex structure at Asn 108. The reaction was monitored by RP-HPLC. Before deglycosylation HPLC trace showed two merged peaks at $t_R = 20.5$ min. After Endo A mediated hydrolysis a single peak at $t_R = 22$ min was observed (Figure 4.13 and 4.14). The de-glycosylated Fabrazyme (dFab 4.7) was then

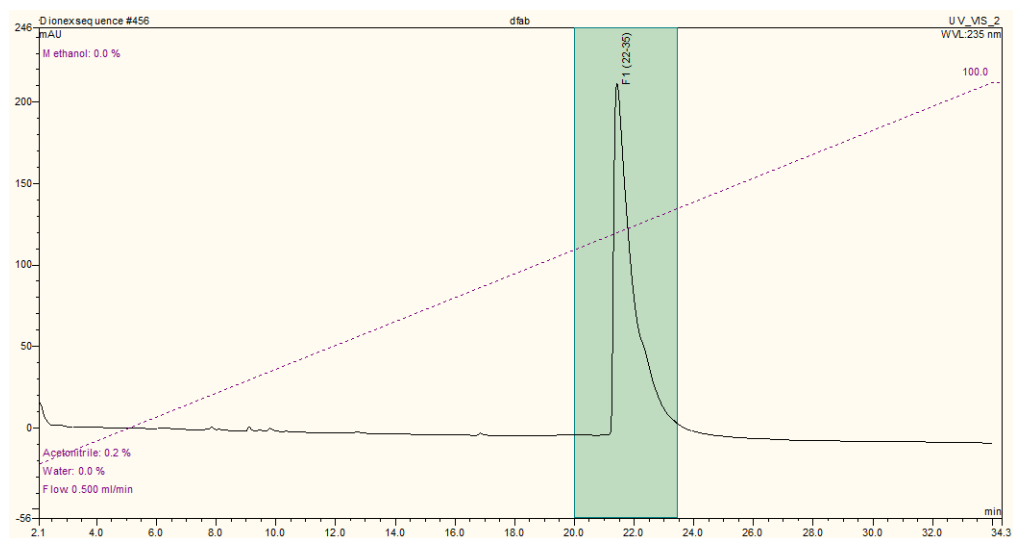
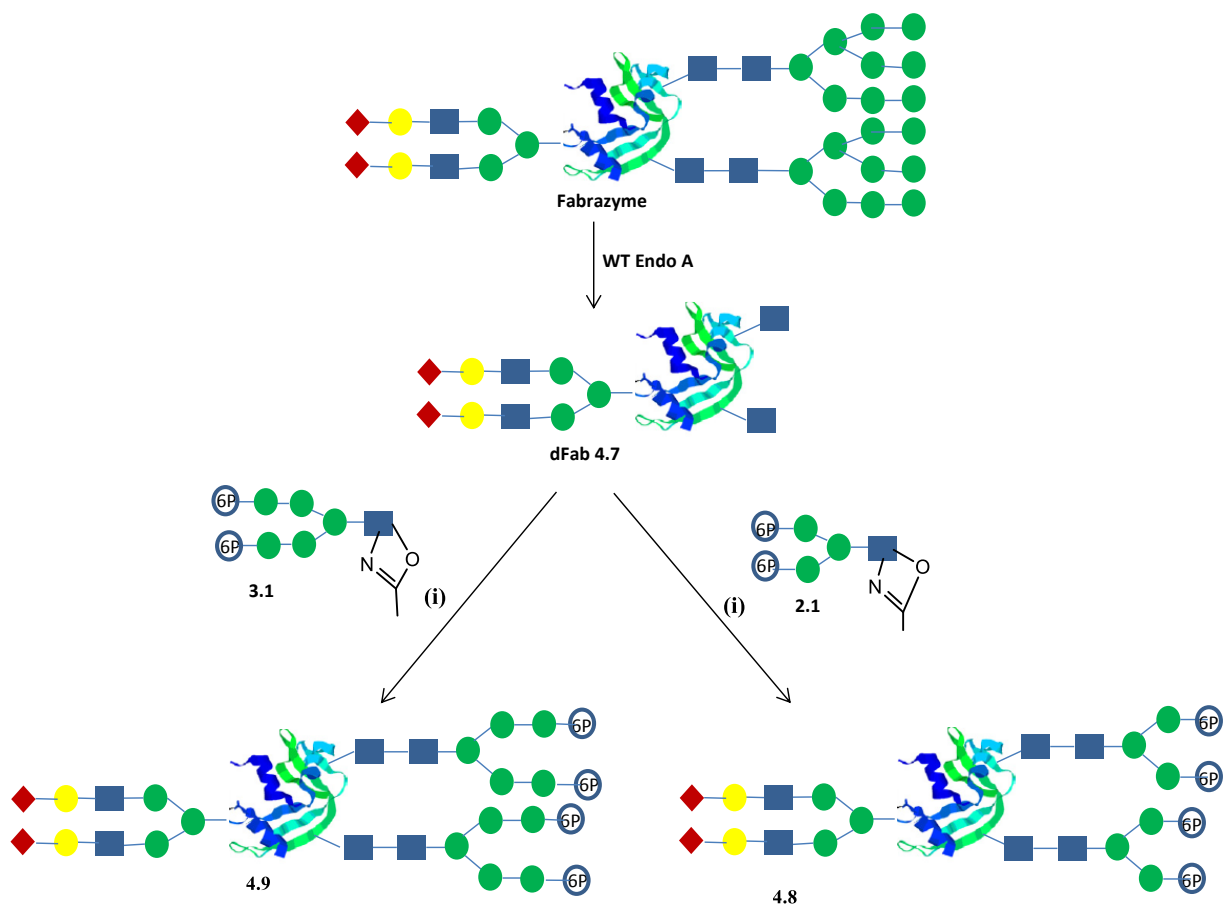


Figure 4.14 HPLC trace of deglycosylated Fabrazyme



Scheme 4.7 Glycoprotein remodeling of Fabrazyme (i) WT Endo A, phosphate buffer pH 6.5, H₂O, 37 °C, 1 h.

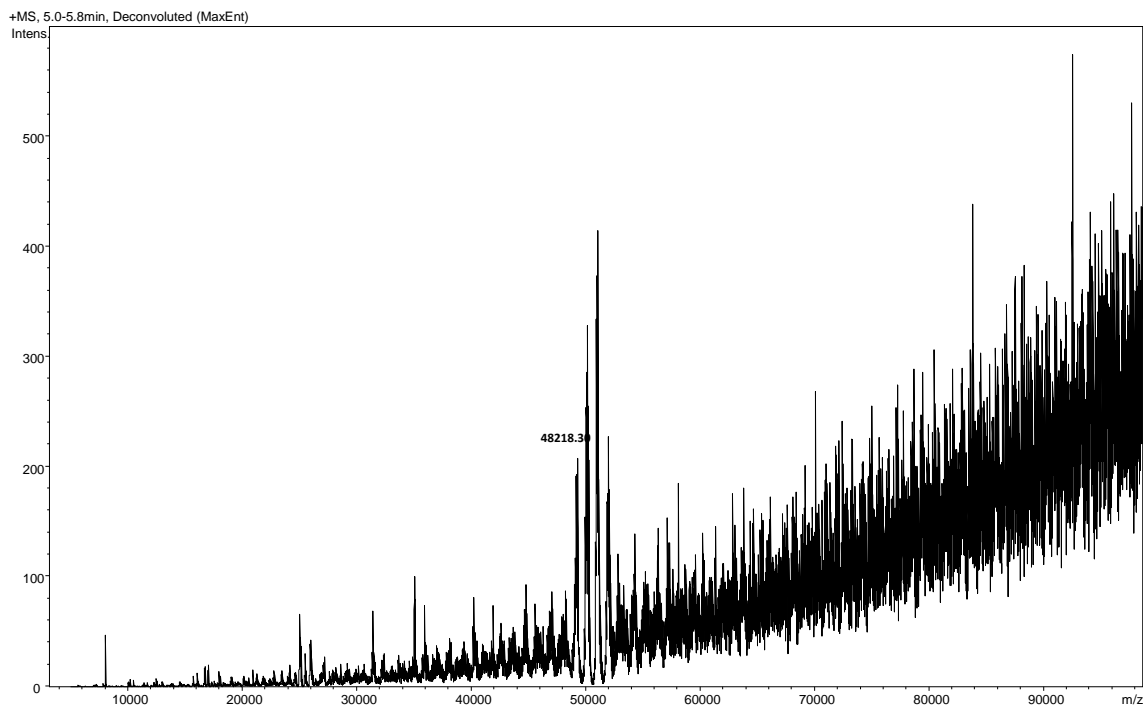


Figure 4.15: HRMS data for 4.8

4.11 Evaluation of binding of 4.5 to the cation-independent mannose-6-phosphate receptor (CI-MPR)

The binding of phosphorylated glycoprotein **4.5** to cation-independent mannose-6-phosphate receptor (CI-MPR) was evaluated using immunocytochemistry and immunoprecipitation methods by Dr Antonia Miller.[‡]

Immunocytochemistry is a technique that is used to anatomically localize the presence of a specific protein or antigen in the cells by use of a specific primary antibody that binds to it. The primary antibody is then bound by a secondary antibody that has a conjugated

[‡] This work was performed by Dr Antonia Miller, Callaghan Innovation Research Ltd.

fluorophore, which allows the visualization of the protein under a fluorescence microscope.

In order to perform the CI-MPR binding studies with glycoprotein **4.5**, liver carcinoma cells (HepG2) were cultured on chamber slides in the presence or absence of 1 μ M (M6P)₂RNase for 2 hours under standard cell culture conditions (37 °C, 5% CO₂, minimal essential media, 1.5% BSA, 100 units/ml penicillin, 100 μ g/ml streptomycin, 250 ng/ml amphotericin). HepG2 cells were also incubated in parallel with 1 μ M deglycosylated RNase B to provide a control for MPR-independent RNase binding. After incubation, the cells were fixed (4% paraformaldehyde in 1 x phosphate buffered saline, PBS) and permeabilized (80% methanol in 1x PBS). The cells were then blocked with 3% non-fat dried milk/1% BSA in 1x PBS, and two primary antibodies were then applied to label the CI-MPR (mouse anti-cation independent mannose-6-phosphate receptor) and RNase (rabbit anti-RNase A). Cells were then incubated with fluorophore-conjugated secondary antibodies which allow for visualization of CI-MPR and RNase (goat anti-mouse IgG-Alexa Fluor 488 and goat anti-rabbit IgG-Alexa Fluor 568 respectively) through their interaction with previously applied primary antibodies. The cells were then stained with a nuclear stain (4',6-diamidino-2-phenylindole, DAPI) and mounted with coverslips. The Images were captured on a Leica TCS SP5 confocal microscope using a 63x objective lens (Figures 4.16). Appropriate controls were included which did not receive either primary or secondary antibody or both to control for non-specific binding/background fluorescence. It depicts the co-localization of (M6P)₂RNase with CI-MPR in HepG2 cells following 2 hours of incubation with 1 μ M (M6P)₂RNase. Staining of

HEPG2 cells post incubation for **A**, the nuclear stain DAPI, **B**, the CI-MPR, **C**, RNase , **D**, Merged image of **A-C**, indicate co-localization of CI-MPR with RNase.

The co-localization of the CI-MPR and RNase signals on HepG2 cells demonstrates that a phosphorylated glycoprotein or glycopeptide can be specifically targeted to cells expressing cation independent mannose-6-phosphate receptors.

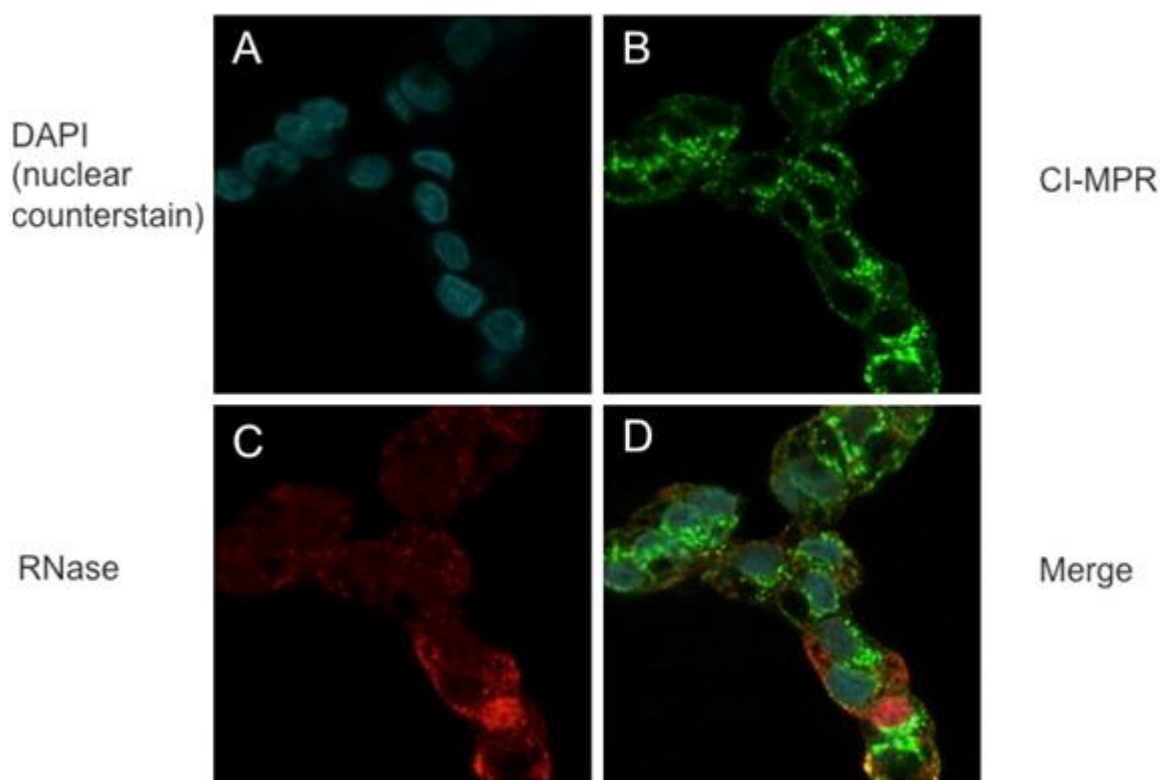


Figure 4.16 Co-localization of the CI-MPR and RNase

Immunoprecipitation is a technique used to isolate or detect a particular protein from a sample containing a mixture of proteins. In this technique, the particular protein of

interest is precipitated out using an antibody that specifically binds to that protein. This method requires the antibody to be coupled to a solid substrate.

HepG2 cells were treated with either **4.5** or deglycosylated RNase as above and cell lysates prepared. The cell lysates were then incubated with mouse anti-CI-MPR antibody-conjugated Protein A sepharose overnight at 4 °C to capture (M6P)₂RNase (**4.5**) bound to the CI-MPR. The protein A sepharose was washed extensively and the bound sample containing (M6P)₂RNase-CI-MPR was then eluted with reducing buffer containing sodium dodecyl sulphate and dithiothreitol. The eluate was subjected to Western blotting for detection of RNase captured by CI-MPR (Figure 4.17).

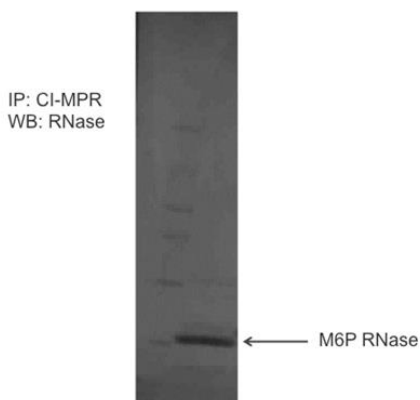


Figure 4.17 Western blotting (RNase capture by CI-MPR)

4.12 Conclusions

This chapter described the ability of endohexosaminidases to utilise phosphorylated *N*-glycan oxazolines **2.1** and **3.1** as donor substrates for a series of enzyme catalyzed

glycosylation reactions. It was shown that, although Endo A could process the tetrasaccharide **2.1**, Endo M only resulted in hydrolysis of the oxazoline **2.1**. It was also interesting to observe that the hydrolysis of the pentasaccharide product formed using WT Endo A was very slow, indicating a significant kinetic window for the synthetic reaction. Both Endo A and Endo M were able to process the phosphorylated hexasaccharide oxazoline **3.1**, albeit giving products in a lower overall yield. From the literature it is known that, *N*-glycans up to a tetrasaccharide in size are only cleaved slowly by ENGases. However, as the size of *N*-glycan is increased, product hydrolysis becomes more rapid.²¹⁵

We have also demonstrated the glycosylation of dRNase B with a phosphorylated tetrasaccharide oxazoline **2.1** to obtain a neoglycoprotein in modest yield. Furthermore, the same approach was successfully employed for the glycosylation of dRNase with a full length phosphorylated *N*-glycan hexasaccharide **3.1** to obtain a glycoprotein, the glycan of which has been optimized for binding to M6PRs.

The ultimate objective of this work was to develop methodology to access homogeneous phosphorylated glycoenzymes to optimize the treatment of lysosomal storage diseases by ERT. In the case reported here, we successfully deglycosylated a commercially available drug, Fabrazyme, which is clinically used for the treatment of Fabry disease, and glycosylated it with both tetrasaccharide **2.1** and hexasaccharide **3.1**, respectively, to obtain two different phosphorylated glycoenzymes.

4.13 Future work

This work has demonstrated the ability of ENGases to process phosphorylated *N*-glycan oxazolines, and the use of a chemo-enzymatic method to access homogeneous phosphorylated glycoproteins. At this point of time, the successful purification of the glycoproteins has yet not been achieved. Further work must be carried to develop a purification method for these glycoenzymes.

Furthermore, additional work must be carried out to synthesize various different sized phosphorylated oxazoline donors both to elucidate the substrate specificity of the ENGases and to synthesize optimal homogeneous glycoprotein products. These homogeneous glycoproteins may then be used to optimise the treatment of a variety of lysosomal storage diseases by ERT.

Chapter 5

Experimental Section

5.1 General chemical procedures

Unless otherwise stated, reagents were obtained from commercial sources and used as received. Water was purified by reverse osmosis *in-house*. HPLC-grade solvents were used for reactions and in case of moisture-sensitive reactions; solvents were dried by literature procedures and freshly distilled as required. Melting points were recorded on an Electrothermal melting point apparatus. Elemental analysis was done by the Campbell Microanalytical Laboratory, University of Otago. Thin Layer Chromatography (t.l.c.) was carried out on Merck Kieselgel 60F₂₅₄ pre-coated glass-backed plates. Visualisation of the plates was achieved using a UV lamp ($\lambda_{\text{max}} = 254$ or 365 nm), and/or ammonium molybdate (5% in 2 M sulfuric acid), or sulfuric acid (5% in ethanol). Flash column chromatography was carried out using Sorbsil C60 40/60 silica.

Nuclear Magnetic Resonance

¹H and ¹³C NMR spectra were recorded on Agilent 400-MR and Varian 500 INOVA instruments operating for ¹H NMR at 400 and 500 MHz, respectively and at 100 and 125 MHz, respectively, for ¹³C NMR. All the ¹H NMR spectra recorded in deuterated solvents were referenced to the solvent peak and/or TMS: CDCl₃, 7.26 ppm; CD₃CN, 2.0 ppm; CD₃OD, 3.3 ppm; DMSO, 2.6 ppm. ¹³C NMR were all referenced to their solvent peaks: chloroform, 77.0 ppm; acetonitrile, 36.8 ppm; methanol, 49.3 ppm; DMSO, 39.6 ppm. When required, gCOSY, 1-D TOCSY, HSQC, HSQC “non-decoupled” and HMBC experiments were performed using standard pulse sequences.

Mass Spectrometry

Mass spectra were recorded by Dr. Marie Squire and Dr. Alexander on either a DIONEX Ultimate 3000 or Bruker MaXis 4G spectrometer, operated in high resolution positive ion electrospray mode. Samples were prepared by dissolving in an appropriate solvent at the required concentration.

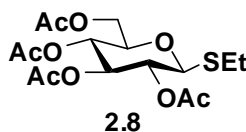
Infrared Spectroscopy

Infrared spectra were recorded on a Perkin–Elmer Spectrum One FTIR instrument operating in diffuse reflectance mode with samples prepared as KBr pellets (KBr) or on a Bruker FTIR spectrometer with Alpha's Platinum ATR single reflection diamond where the neat samples were recorded.

Carbohydrates and derivatives have been named in accordance with IUPAC recommendations and numbered according to the carbohydrate convention. The two protons on C-6 are labelled H-6 and H-6'.

5.2 Experimental for chapter 2

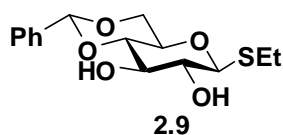
Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-glucopyranoside 2.8



D-Glucose pentaacetate (5.0 g, 12.8 mmol) and ethanethiol (1.39 mL, 19.2 mmol) were dissolved in anhydrous DCM (20 mL). Boron trifluoride diethyl etherate (2.40 mL, 19.2 mmol) was added to the solution and the mixture stirred at rt under an atmosphere of nitrogen. After 4 h t.l.c. (petrol:ethyl acetate 1:1) indicated formation of a single product

(Rf 0.55) and complete consumption of the starting material (Rf 0.45). The reaction mixture was quenched with triethylamine (2.5 mL), poured onto water (40 mL) and extracted into DCM (3 x 50 mL). The combined organics were washed with sodium hydroxide (2 x 50 mL of a 1 M solution) and brine (50 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was crystallised using petroleum ether to afford ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-glucopyranoside **2.8** (3.0 g, 60%) as a white crystalline solid, m.p. 81-83 °C [lit. 82];¹⁴¹ [α]_D²⁰ -30 (c, 1.0 in CHCl₃) [Lit. [α]_D²⁰ -26 (c, 1.0 in CHCl₃)];¹⁴¹ δ _H (500 MHz, CDCl₃) 1.28 (1H, t, *J* 7.5 Hz, CH₂CH₃), 2.02, 2.03, 2.07, 2.08 (12H, 4 x s, 4 x CH₃), 2.69-2.73 (2H, m, CH₂CH₃), 3.72 (1H, m, H-5), 4.14 (1H, dd, *J*_{6,6'} 12.3 Hz, H-6), 4.25 (1H, dd, H-6'), 4.50 (1H, d, *J*_{1,2} 10.2 Hz, H-1), 5.04 (1H, at, *J* 9.6 Hz, H-2), 5.09 (1H, at, *J* 10.2 Hz, H-4), 5.23 (1H, at, *J* 9.4 Hz, H-3).

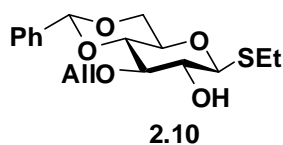
Ethyl 4,6-*O*-benzylidene-1-thio- β -D-glucopyranoside **2.9**



Sodium methoxide (0.01 g, 0.13 mmol) was dissolved in methanol (6 mL) and the solution cooled to rt. Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-glucopyranoside **2.8** (0.50 g, 1.27 mmol) was added in portions and the mixture stirred at rt under an atmosphere of nitrogen. After 1 h t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a single product (Rf 0.0) and complete consumption of starting material (Rf 0.55). The mixture was concentrated *in vacuo*. DMF (6 mL), benzaldehyde dimethyl acetal (0.38 mL, 2.54 mmol) and DL-camphor-10-sulfonic acid (0.058 g, 0.25 mmol) were added and the mixture rotated in a round bottom flask on a rotary evaporator at 60 °C under a pressure

of 240 mbar. After 4 h t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.25). The reaction mixture was quenched with triethylamine and concentrated *in vacuo*. The residue was dissolved in ethyl acetate (10 mL), washed with water (2 x 10 mL) and brine (2 x 10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was crystallised (petrol/ethyl acetate) to afford ethyl 4,6-*O*-benzylidene-1-thio- β -D-glucopyranoside **2.9** (0.24 g, 65% over two steps) as a white crystalline solid, m.p. 131-133 °C [lit. 129-130];¹⁴¹ $[\alpha]_D^{20}$ -58 (*c*, 1.0 in $CHCl_3$) [lit. $[\alpha]_D^{22}$ -63 (*c*, 1.05 in $CHCl_3$)];¹⁴¹ δ_H (500 MHz, $CDCl_3$) 1.34 (3H, t, *J* 7.5 Hz, CH_2CH_3), 2.74-2.81 (2H, m, CH_2CH_3), 3.49-3.54 (2H, m, H-2, H-5), 3.59 (1H, at, *J* 8.8 Hz, H-3), 3.77 (1H, at, *J* 10.1 Hz, H-6), 3.85 (1H, at, *J* 8.9 Hz, H-4), 4.36 (1H, dd, $J_{5,6}$ 4.8 Hz, $J_{6,6'}$ 10.6 Hz, H-6'), 4.48 (1H, d, $J_{1,2}$ 9.6 Hz, H-1), 5.55 (1H, s, $PhCH(O)$), 7.37-7.51 (5H, m, 5 x Ar-H).

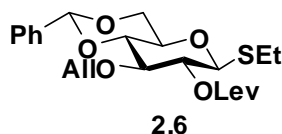
Ethyl 3-*O*-allyl-4,6-*O*-benzylidene-1-thio- β -D-glucopyranoside **2.10**



Ethyl 4,6-*O*-benzylidene-1-thio- β -D-glucopyranoside **2.9** (10.0 g, 32 mmol) was dissolved in methanol (200 mL) and dibutyltin oxide (9.56 g, 38.4 mmol) added. The slurry was stirred at reflux under an nitrogen atmosphere for 3 h and the resulting solution concentrated *in vacuo*. DMF (100 mL), allyl bromide (4.97 mL, 57.6 mmol) and cesium fluoride (6.31 g, 41.6 mmol) were added to the residue and the mixture stirred at rt under an atmosphere of nitrogen. After 3 days t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.70) and a trace of remaining starting material (R_f

0.25). The reaction mixture was concentrated *in vacuo*, the residue dissolved in DCM (100 mL), washed with 1N HCl solution (100 mL). Washed the organics with DI water (100 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford ethyl 3-*O*-allyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside **2.10** (7.0 g, 63% over two steps) as an amorphous white solid, m.p. 135-137 °C [lit. 137-141 °C]; ¹⁴¹ [α]_D²⁰ -47 (c, 1.0 in CHCl₃) [lit. [α]_D¹⁹ -53 (c, 1.1 in CHCl₃)]; ¹⁴¹ δ_H (500 MHz, CDCl₃) 1.33 (3H, t, *J* 7.5 Hz, CH₂CH₃), 2.5 (1H, d, *J* 2.0 Hz, OH), 2.77 (2H, m, CH₂CH₃), 3.47-3.66 (4H, m, H-2, H-3, H-4, H-5), 3.76 (1H, at, *J* 10.3 Hz, H-6), 4.30 (1H, dd, *J*_{vic} 6.1 Hz, *J*_{gem} 12.6 Hz, OCHH'CH=CH₂), 4.36 (1H, dd, *J*_{5,6'} 4.8 Hz, *J*_{6,6'} 10.6 Hz, H-6'), 4.45 (1H, m, OCHH'CH=CH₂), 4.49 (1H, d, *J*_{1,2} 9.5 Hz, H-1), 5.20 (1H, dd, *J*_{vic} 10.2 Hz, CH=CHH_Z), 5.32 (1H, dd, *J*_{vic} 17.1 Hz, *J*_{gem} 1.7 Hz, CH=CHH_E), 5.55 (1H, s, PhCH(O)), 5.97 (1H, m, OCH₂CH=CH₂), 7.26-7.49 (5H, m, 5 x Ar-H).

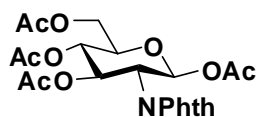
Ethyl 3-*O*-allyl-4,6-*O*-benzylidene-2-*O*-levulinoyl-1-thio-β-D-glucopyranoside **2.6**



Ethyl 3-*O*-allyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside **2.10** (6.0 g, 17 mmol) was dissolved in DCM (240 mL). Levulinic acid (3.46 mL, 34 mmol), dicyclohexylcarbodiimide (7.0 g, 34 mmol) and DMAP (0.21 g, 1.7 mmol) were added and the mixture was stirred at rt under an atmosphere of nitrogen. After 21 h t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of a single product (R_f 0.60) and complete consumption of **2.1** (R_f 0.55). Filtered the reaction mixture and washed the residue with

DCM. The filtrate was diluted with DCM (300 mL), washed with water (2 x 500 mL), sodium hydrogen carbonate (2 x 500 mL of a saturated aqueous solution) and brine (500 mL), dried (MgSO₄), filtered, concentrated *in vacuo* and the residue purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford ethyl 3-*O*-allyl-4,6-*O*-benzylidene-2-*O*-levulinoyl-1-thio-β-D-glucopyranoside **2.6** (6.44 g, 84%) as an amorphous white solid, m.p. 97.5-98.5 °C [lit. 98-100 °C];¹⁴¹ m.p. [α]_D²⁰ – 60.4 (c, 1.0 in CHCl₃); [lit. [α]_D¹⁸ – 51 (c, 0.5 in CHCl₃)];¹⁴¹ δ _H (500 MHz, CDCl₃) 1.26 (3H, t, *J* 7.5 Hz, CH₂CH₃), 2.17 (3H, s, CH₃), 2.64-2.84 (6H, m, 3 x CH₂), 3.47-3.51 (1H, m, H-5), 3.67-3.71 (2H, m, H-3, H-4), 3.78 (1H, at, *J* 10.3 Hz, H-6), 4.17 (1H, dd, *J*_{vic} 6.0 Hz, *J*_{gem} 13.0 Hz, OCHH'CH=CH₂), 4.34-4.48 (2H, m, H-6', OCHH'CH=CH₂), 4.50 (1H, d, *J*_{1,2} 9.1 Hz, H-1), 5.02 (1H, at, *J* 9.9 Hz, H-2), 5.16 (1H, d, *J*_{vic} 10.4 Hz, CH=CHH_Z), 5.25 (1H, dd, *J*_{gem} 1.7 Hz, *J*_{vic} 17.2 Hz, CH=CHH_E), 5.56 (1H, s, PhCH(O)), 5.88 (1H, m, CH=CH₂), 7.36-7.36 (3H, m, 3 x Ar-H), 7.47-7.49 (2H, m, 2 x Ar-H).

1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.11**

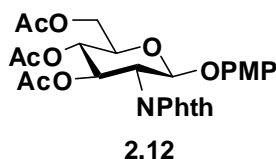


2.11

Sodium methoxide (13.8 g, 254.9 mmol) was added to a stirred mixture of D-Glucosamine hydrochloride (50 g, 231.8 mmol) in MeOH (1.25 L) and stirred under an atmosphere of nitrogen for 1 h. Phthalic anhydride (34.33 g, 231.8 mmol) and triethylamine (64.6 mL, 464 mmol) were added and the mixture stirred under an atmosphere of nitrogen. After 3 days the reaction mixture was concentrated *in vacuo*, the

residue dissolved in pyridine (700 mL) and cooled to 0 °C. Acetic anhydride (650 mL) was added portionwise, the reaction mixture allowed to warm to rt and stirred for 22 h. After this time t.l.c. (petrol:ethyl acetate, 1:2) indicated complete consumption of starting material (R_f 0.0) and formation of a major product (R_f 0.30). The reaction mixture was poured onto ice/water (1 L) and extracted with DCM (4 x 500 mL). The organics were washed with HCl (4 x 500 mL of a 1 M solution), sodium hydrogen carbonate (2 x 500 mL of a saturated solution), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) and crystallisation (petrol/diethyl ether) to afford 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.11** (45 g, 50% over three steps) as a white solid, m.p. 79.5-82 °C [lit. 78-81];¹⁴¹ $[\alpha]_D^{20} + 110$ (*c*, 1.0 in $CHCl_3$) [lit. $[\alpha]_D^{22} + 64$ (*c*, 1.0 in $CHCl_3$)];¹⁴¹ δ_H (500 MHz, $CDCl_3$) 1.88, 2.00, 2.05, 2.10 (12H, 4 x s, 4 x CH_3), 4.00 (1H, ddd, $J_{4,5}$ 10.2 Hz, $J_{5,6}$ 2.2 Hz, $J_{5,6'}$ 4.5 Hz, H-5), 4.15 (1H, dd, $J_{6,6'}$ 12.5 Hz, H-6), 4.38 (1H, dd, H-6'), 4.51 (1H, dd, $J_{1,2}$ 8.9 Hz, $J_{2,3}$ 10.5 Hz, H-2), 5.22 (1H, dd, $J_{3,4}$ 9.2 Hz, H-4), 5.85 (1H, m, H-3), 6.51 (1H, d, H-1), 7.73-7.87 (4H, m, 4 x Ar-H).

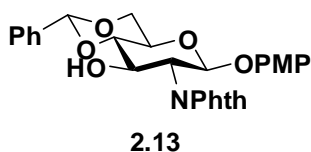
***p*-Methoxyphenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranoside**
2.12



1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.11** (2.0 g, 2.10 mmol) was dissolved in DCM (12 mL) and *para*-methoxyphenol (0.78 g, 3.15 mmol) added. The mixture was stirred at rt under an atmosphere of nitrogen for 5 min; after this

time boron trifluoride diethyl etherate (0.76 mL, 3.15 mmol) was added portionwise. After 21 h t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (Rf 0.60) and complete consumption of starting material (Rf 0.55). The reaction mixture was poured onto sodium hydrogen carbonate (12 mL of a saturated aqueous solution), extracted into DCM (3 x 12 mL), washed with sodium hydrogen carbonate (15 mL of a saturated aqueous solution), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was crystallised (petrol/ethyl acetate) to afford *p*-methoxyphenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.3** (1.6 g, 71%) as a white amorphous solid, m.p. 139-142 °C [lit. 148-149];¹⁴¹ $[\alpha]_D^{20} + 53.2$ (c, 1.0 in CHCl₃) [lit. $[\alpha]_D^{25} + 51$ (c, 1.0 in CHCl₃)];¹⁴¹ δ_H (500 MHz, CDCl₃) 1.89, 2.05, 2.11 (9H, 3 x s, 3 x CH₃), 3.73 (3H, s, OCH₃), 3.95-3.99 (1H, m, H-5), 4.19 (1H, dd, $J_{5,6}$ 2.3 Hz, $J_{6,6'}$ 12.2 Hz, H-6), 4.34 (1H, dd, $J_{5,6'}$ 5.1 Hz, H-6'), 4.59 (1H, dd, $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 10.5 Hz, H-2), 5.24 (at, J 9.8 Hz, H-4), 5.86 (1H, dd, $J_{3,4}$ 9.9 Hz, H-3), 5.86 (1H, d, H-1), 6.73-6.86 (4H, m, 4 x Ar-H), 7.74-7.87 (4H, m, 4 x Ar-H).

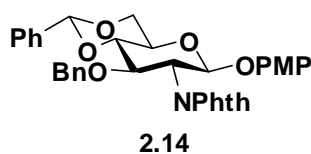
p*-Methoxyphenyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.13*



To a solution of *p*-methoxyphenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.3** (1.3 g, 2.4 mmol) in MeOH (17 mL) was added NaOMe (0.013 g, 0.24 mmol) in portions and the mixture stirred at rt under an atmosphere of nitrogen. After 17 h t.l.c. (Petrol:ethyl acetate, 1:1) indicated formation of a single product (Rf 0.0)

and complete consumption of starting material (Rf 0.60). The mixture was concentrated *in vacuo*. DMF (13 mL), benzaldehyde dimethyl acetal (0.71 mL, 4.8 mmol) and DLeamphor-10-sulfonic acid (0.12 g, 0.48 mmol) were added to the residue and the mixture rotated in a round bottom flask on a rotary evaporator at 60 °C under a pressure of 240 mbar. After 5 h t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (Rf 0.65). The reaction mixture was concentrated *in vacuo*, the residue dissolved in ethyl acetate (20 mL), washed with water (2 x 20 mL) and brine (20 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was crystallised (petrol/ethyl acetate) to afford *p*-methoxyphenyl 4,6-*O*-benzylidene- 2-deoxy-2-phthalimido-β-D-glucopyranoside **2.13** (0.65 g, 54% over two steps) as an amorphous white solid, m.p. 125-127 °C [lit. 128-129];¹⁴¹ $[\alpha]_D^{20} + 11$ (c, 1.0 in CHCl₃) [lit. $[\alpha]_D^{25} + 9.7$ (c, 0.8 in CHCl₃)];¹⁴¹ δ_H (500 MHz, CDCl₃) 2.47 (1H, br s, OH), 3.70-3.79 (2H, m, H-4, H-5), 3.73 (3H, s, OCH₃), 3.86 (1H, at, *J* 9.6 Hz, H-6), 4.40 (1H, dd, *J*_{5,6'} 4.1 Hz, *J*_{6,6'} 10.5 Hz, H-6'), 4.51 (1H, dd, *J*_{1,2} 8.8 Hz, *J*_{2,3} 10.5 Hz, H-2), 4.73 (1H, at, *J* 8.9 Hz, H-3), 5.60 (1H, s, PhCH(O)), 5.82 (1H, d, H-1), 6.73- 6.86 (4H, m, 4 x Ar-H), 7.38-7.51 (5H, m, 5 x Ar-H), 7.72-7.87 (4H, m, 4 x Ar-H).

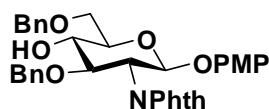
***p*-Methoxyphenyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β D-glucopyranoside 2.14**



A solution of *p*-methoxyphenyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β D-glucopyranoside **2.13** (0.50 g, 0.99 mmol) in DMF (10 mL) was cooled to 0 °C. Sodium

hydride (50% dispersion in mineral oil, 0.07 g, 1.5 mmol) was added in portions to the cooled solution. Once addition was complete the slurry was left to stir for 30 min at 0 °C under an atmosphere of nitrogen, before the dropwise addition of benzyl bromide (0.18 mL, 1.5 mmol). The reaction mixture was allowed to warm gradually to rt. After 17 h t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of product (Rf 0.50) and complete consumption of starting material (Rf 0.25). The reaction was quenched with methanol (2 mL), concentrated *in vacuo* and diluted with ethyl acetate (10 mL). The solution was washed with water (2 x 10 mL), the washings re-extracted with ethyl acetate (2 x 10 mL) and the combined organics dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified twice by flash column chromatography (petrol:ethyl acetate, 2:1 and toluene:ethyl acetate, 9:1) to afford *p*-methoxyphenyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.14** (0.4 g, 83%) as a white crystalline solid, m.p. 120-122 °C [lit. 118-119];¹⁴¹ $[\alpha]_{\text{D}}^{20} + 81$ (*c*, 1.0 in CHCl₃) [lit. $[\alpha]_{\text{D}}^{25} + 72$ (*c*, 0.8 in CHCl₃)];¹⁴¹ δ_{H} (500 MHz, CDCl₃) 3.72-3.77 (1H, m, H-5), 3.71 (3H, s, OCH₃), 3.87-3.93 (2H, m, H-4, H-6), 4.42 (1H, dd, $J_{5,6} = 4.8$ Hz, $J_{6,6'} = 10.6$ Hz, H-6'), 4.47-4.50 (2H, m, H-2, H-3), 4.54, 4.83 (2H, m, PhCH₂), 5.66 (1H, s, PhCH(O)), 5.74 (1H, d, $J_{1,2} = 8.2$ Hz, H-1), 6.71-6.82 (4H, m, 4 x Ar-H), 6.82-7.03 (5H, m, 5 x Ar-H), 7.40-7.72 (5H, m, 5 x Ar-H), 7.74-7.90 (4H, m, 4 x Ar-H).

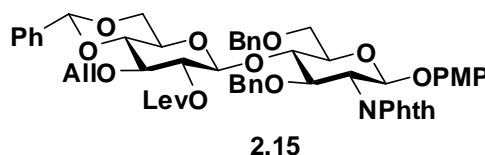
***p*-Methoxyphenyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 2.7**



2.7

To a dry round bottom flask containing activated 4 Å molecular sieves (200 mg), *p*-methoxyphenyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.14** (200 mg, 340 μmol), THF (10 mL) and sodium cyanoborohydride (265 mg, 4.20 mmol) were added. The reaction mixture was cooled down to 0 °C and hydrochloric acid (1 M in diethyl ether) added dropwise until gas ceased to evolve. The reaction mixture was stirred at 0 °C. After 2.5 h, t.l.c. (petrol:ethyl acetate, 3:1) indicated formation of a major product (R_f 0.2) and complete consumption of starting material (R_f 0.35). Water (20 mL) and DCM (20 mL) were added, and the organic layer washed with sodium bicarbonate (2 x 50 mL) and brine (2 x 50 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 3:1) to give *p*-methoxyphenyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **3.8** (141 mg, 70%) as a clear oil. $[\alpha]_D^{20} +63$ (*c*, 1.0 in CDCl₃) [lit. $[\alpha]_D +56.1$ (*c*, 0.8)];²³ δ_H (400 MHz, CDCl₃) 2.91 (1H, d, $J_{3,OH}$ 2.2 Hz, OH), 3.71 (3H, s, OCH₃), 3.72-3.77 (1H, m, H-5), 3.80-3.88 (2H, m, H-6, H-6'), 3.91 (1H, m, H-4), 4.31 (1H, dd, $J_{2,3}$ 10.8 Hz, $J_{3,4}$ 8.3 Hz, H-3), 4.41 (1H, dd, $J_{1,2}$ 8.3 Hz, H-2), 4.57, 4.78 (2H, m, J 12.2 Hz, PhCH₂), 4.59, 4.65 (2H, m, J 11.9 Hz, PhCH₂), 5.67 (1H, d, H-1), 6.68-6.83 (4H, m, 4 x Ar-H), 6.95-7.09 (5H, m, 5 x Ar-H), 7.30-7.39 (5H, m, 5 x Ar-H), 7.70-7.84 (4H, m, 4 x Ar-H).

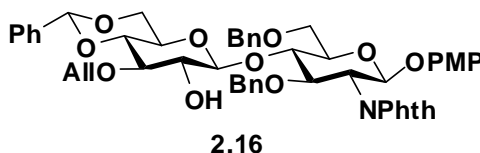
p-Methoxyphenyl 3-*O*-allyl-4,6-*O*-benzylidene-2-*O*-levulinoyl-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.15**



p-Methoxyphenyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.7** (2.21 g, 3.70 mmol), ethyl 3-*O*-allyl-4,6-*O*-benzylidene-2-*O*-levulinoyl-1-thio- β -D-glucopyranoside **2.6** (2.20 g, 4.82 mmol) and tri-*tert*-butylpyrimidine (3.76 g, 15.2 mmol) were dissolved in anhydrous DCM (22 mL) and transferred to a flame-dried round-bottom flask containing activated 3Å molecular sieves (8.0 g). The mixture was cooled to 0 °C under an atmosphere of nitrogen and methyl trifluoromethanesulfonate (1.70 mL, 15.2 mmol) added. After 14 h t.l.c. (petrol:ethyl acetate, 2:1) indicated complete consumption of **2.7** (R_f 0.40) and formation of a major product (R_f 0.10). Triethylamine was added and the reaction mixture stirred for 10 min before being filtered through celite and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford *p*-methoxyphenyl 3-*O*-allyl-4,6-*O*-benzylidene-2-*O*-levulinoyl- β -D-glucopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.15** (3.35 g, 91%) as a white foam, m.p. 78-79 °C [lit⁷⁶⁻⁷⁸]; $[\alpha]_D^{20}$ +36 (*c*, 1.0 in CDCl₃) [lit. $[\alpha]_D$ +31 (*c*, 0.6 in CHCl₃)];²³ δ_H (400 MHz, CDCl₃) 2.23 (3H, s, CH₃), 2.55-2.88 (4H, m, 2 x CH₂), 3.25 (1H, m, H-5b), 3.45-3.52 (2H, m, H-3b, H-6b), 3.58 (1H, at, *J* 9.6 Hz, H-4b), 3.69 (1H, m, H-5a), 3.71 (3H, s, OCH₃), 3.84 (1H, dd, *J*_{5,6} 1.3 Hz, *J*_{6,6'} 11.1 Hz, H-6a), 3.93 (1H, dd, *J*_{5,6'} 3.3 Hz, H-6'a), 4.10 (1H, dd, *J*_{vic} 5.7 Hz, *J*_{gem} 13.0 Hz, OCHH'CH=CH₂), 4.16 (1H, at, *J* 9.2 Hz, H-4a), 4.28 (1H, dd, *J*_{5,6'} 4.9 Hz, *J*_{6,6'} 10.4 Hz, H-6'b), 4.31-4.36 (2H, m, H-3a, OCHH'CH=CH₂), 4.40 (1H, dd, *J*_{1,2} 8.5 Hz, *J*_{2,3} 10.7 Hz, H-2a), 4.44, 4.81 (2H, m, PhCH₂), 4.52, 4.79 (2H, m, PhCH₂), 4.65 (1H, d, *J*_{1,2} 8.0 Hz, H-1b), 4.96 (1H, dd, *J*_{2,3} 8.8 Hz, H-2b), 5.16 (1H, dd, *J*_{vic} 10.4 Hz, *J*_{gem} 1.5 Hz, CH=CHH_Z), 5.24 (1H, dd, *J*_{vic} 17.3 Hz, CH=CHH_E), 5.46 (1H, s, PhCH(O)), 5.63 (1H, d, H-1a), 5.86 (1H, m, CH=CH₂), 6.69-

6.72 (2H, m, 2 x Ar-H), 6.81-6.83 (2H, m, 2 x Ar-H), 6.87-6.93 (3H, m, 3 x Ar-H), 7.02-7.04 (2H, m, 2 x Ar-H), 7.32-7.41 (8H, m, 8 x Ar-H), 7.46-7.48 (2H, m, 2 x Ar-H), 7.68-7.73 (4H, m, 4 x Ar-H).

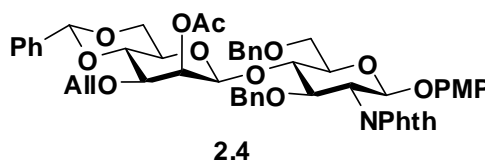
p*-Methoxyphenyl 3-*O*-allyl-4,6-*O*-benzylidene- β -D-glucopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.16*



p-Methoxyphenyl 3-*O*-allyl-4,6-*O*-benzylidene-2-*O*-levulinoyl- β -D-glucopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.15** (0.50 g, 0.5 mmol) was dissolved in DCM (2.5 mL) and methanol (6 mL). Hydrazine acetate (0.115 g, 1.25 mmol) was added and the mixture stirred under an atmosphere of nitrogen. After 14 h t.l.c. (petrol:ethyl acetate, 3:2) indicated complete consumption of starting material (R_f 0.70) and formation of a single product (R_f 0.50). The reaction mixture was concentrated in vacuo and the residue purified by flash column chromatography (2:1 petrol:ethyl acetate) to afford *p*-methoxyphenyl 3-*O*-allyl-4,6-*O*-benzylidene- β -D-glucopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.16** (0.39 g, 87%) as a white foam, m.p. 75-77 °C [lit. 80-83];²³ [α]_D²⁰ + 55 (c, 1.0 in CHCl₃) [lit. [α]_D¹⁹ + 33 (c, 0.3 in CHCl₃)];²³ δ H (500 MHz, CDCl₃) 3.24 (1H, m, H-5b), 3.45-3.57 (4H, m, H-2b, H-3b, H-4b, H-6b), 3.72 (3H, s, OCH₃), 3.74-3.77 (1H, m, H-5a), 3.88 (1H, dd, J_{5,6} 1.7 Hz, J_{6,6'} 11.3 Hz, H-6a), 4.06 (1H, dd, J_{5,6'} 3.6 Hz, H-6'a), 4.17-4.22 (2H, m, H-4a, H-6'b), 4.26 (1H, dd, J_{vic} 6.1 Hz, J_{gem} 12.8 Hz, OCHH'CH=CH₂),

4.42- 4.46 (3H, m, H-2a, H-3a, OCHH'CH=CH₂), 4.45, 4.82 (2H, m, PhCH₂), 4.61, 4.75 (2H, m, PhCH₂), 4.69 (1H, d, $J_{1,2}$ 7.0 Hz, H-1b), 5.21 (1H, dd, J_{vic} 10.4 Hz, J_{gem} 1.5 Hz, CH=CHH_Z), 5.32 (1H, dd, J_{vic} 17.3 Hz, CH=CHH_E), 5.47 (1H, s, PhCH(O)), 5.61 (1H, d, H-1a), 5.96 (1H, m, CH=CH₂), 6.69-6.72 (2H, m, 2 x Ar-H), 6.80-6.83 (2H, m, 2 x Ar-H), 6.88-6.95 (3H, m, 3 x Ar-H), 7.03-7.05 (2H, m, 2 x Ar-H), 7.36-7.40 (8H, m, 8 x Ar-H), 7.46-7.47 (2H, m, 2 x Ar-H), 7.68 (4H, br s, 4 x Ar-H).

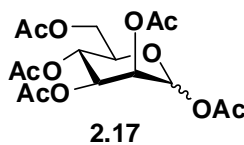
p*-Methoxyphenyl 2-*O*-acetyl-3-*O*-allyl-4,6-*O*-benzylidene-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.4*



p-Methoxyphenyl 3-*O*-allyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl- 2-deoxy-2-phthalimido-β-D-glucopyranoside **3.2** (0.50 g, 0.56 mmol) was dissolved in DCM (20 mL) and the solution cooled to 0 °C. Pyridine (0.81 mL, 10.1 mmol) was added and the mixture stirred for 5 min under an atmosphere of nitrogen; after this time trifluoromethane sulfonic anhydride (0.74 mL, 4.48 mmol) was added and the mixture allowed to warm to room temperature. After 3 h t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of a single product (R_f 0.35) and complete consumption of starting material (R_f 0.10). The reaction mixture was diluted with DCM (15 mL), washed with sodium hydrogen carbonate (2 x 10 mL of a saturated aqueous solution), dried (MgSO₄) and concentrated *in vacuo*. The residue was dissolved in toluene (20 mL) and tetrabutylammonium acetate (1.2 g, mmol) added. The reaction mixture was subjected to

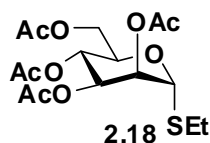
sonication under an atmosphere of nitrogen. After 16 h t.l.c. (petrol:ethyl acetate, 3:2) indicated formation of a single product (Rf 0.45) and complete consumption of the intermediate triflate (Rf 0.55). The mixture was concentrated *in vacuo* and the residue purified by flash column chromatography (3:2 petrol:ethyl acetate) to afford *p*-methoxyphenyl 2-*O*-acetyl-3-*O*-allyl- 4,6-*O*-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.4** (0.40 g, 77% over two steps) as a pale yellow solid, m.p. 79-82 °C [lit. 76-78];²³ $[\alpha]_{\text{D}}^{20} + 36$ (c, 1.0 in CHCl₃) [lit. $[\alpha]_{\text{D}}^{19} + 28.1$ (c, 0.7 in CHCl₃)];²³ δ_{H} (500 MHz, CDCl₃) 2.17 (3H, s, CH₃), 3.21 (1H, m, H-5b), 3.43 (1H, dd, $J_{2,3}$ 2.4 Hz, $J_{3,4}$ 8.9 Hz, H-3b), 3.59 (1H, at, J 10.3 Hz, H-6b), 3.67 (1H, br d, J 9.9 Hz, H-5a), 3.72 (3H, s, OCH₃), 3.77-3.88 (3H, m, H-4b, H-6a, H-6'a), 4.04-4.07 (2H, m, OCHH'CH=CH₂, OCHH'CH=CH₂), 4.18-4.23 (2H, m, H-4a, H-6'b), 4.32 (1H, at, J 9.0 Hz, H-3a), 4.39-4.45 (2H, m, H-2a, PhCH₂), 4.54, 4.80 (2H, m, PhCH₂), 4.74 (1H, br s, H-1b), 4.86 (1H, d, J 12.3 Hz, PhCH₂), 5.18 (1H, dd, J_{vic} 10.3 Hz, J_{gem} 1.3 Hz, CH=CHH_Z), 5.30 (1H, dd, J_{vic} 17.4 Hz, CH=CHH_E), 5.41 (1H, d, J 3.4 Hz, H-2b), 5.51 (1H, s, PhCH(O)), 5.61 (1H, d, $J_{1,2}$ 8.2 Hz, H-1a), 5.85 (1H, m, CH=CH₂), 6.69-6.72 (2H, m, 2 x Ar-H), 6.80-6.82 (2H, m, 2 x Ar-H), 6.89-6.95 (3H, m, 3 x Ar-H), 7.01-7.04 (2H, m, 2 x Ar-H), 7.32-7.39 (8H, m, 8 x Ar-H), 7.46-7.48 (2H, m, 2 x Ar-H), 7.68 (4H, br s, 4 x Ar-H).

1,2,3,4,6-Penta-*O*-acetyl-mannopyranose **2.17**



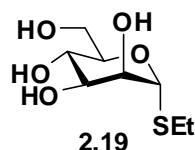
D (+)-Mannose (15.0 g, 83.2 mmol) was dissolved in pyridine (300 mL), the reaction mixture cooled to 0 °C and acetic anhydride (191 mL) was added slowly. The reaction mixture was stirred and allowed to warm to room temperature. After 20 h, t.l.c. (ethyl acetate) indicated formation of a major product (R_f 0.7) and complete consumption of starting material (R_f 0). The reaction mixture was diluted with DCM (50 mL) and quenched by addition of sodium thiosulphate (100 mL) 10% in aqueous solution. The reaction mixture was diluted with Separated the organic layer and aqueous layer extracted with DCM (2 x 50 mL). The organic layers were combined and washed with NaHCO_3 (2 x 100 mL of a satd solution), brine (2 x 100 mL), dried (Na_2SO_4), filtered and concentrated *in vacuo* to give 1,2,3,4,6-penta-*O*-acetyl-D-mannopyranoside **2.17** (32.0 g, 98.5%) as a pale oil and mixture of anomers (7:1, α : β). δ_{H} (500 MHz, CDCl_3)²³ α anomer: 2.01, 2.05, 2.09, 2.17, 2.18 (15H, 5 x s, 5 x CH_3), 4.05-4.07 (1H, m, H-5), 4.08-4.11 (1H, m, H-6), 4.28 (1H, dd, $J_{5,6'}$ 4.8 Hz, $J_{6,6'}$ 12.4 Hz, H-6'), 4.33 (1H, m, H-2), 5.32-5.38 (2H, m, H-3, H-4), 6.08 (1H, br. s, H-1); β anomer: 2.01, 2.05, 2.09, 2.17, 2.18 (15H, 5 x s, 5 x CH_3), 3.81 (1H, m, H-5), 4.11-4.15 (1H, m, H-6), 4.29-4.33 (1H, m, H-6'), 5.13 (1H, dd, $J_{2,3}$ 3.2 Hz, $J_{3,4}$ 9.4 Hz, H-3), 5.32 (1H, m, H-4), 5.48-5.49 (1H, m, H-2), 5.86 (1H, br. s, H-1).

Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio- α -D-mannopyranoside 2.18



1,2,3,4,6-penta-*O*-acetyl-D-mannopyranoside **2.17** (30.0 g, 76.8 mmol) and ethanethiol (8.33 mL, 115.3 mmol) were dissolved in anhydrous DCM (300 mL). Boron trifluoride diethyl etherate (14.2 mL, 115.3 mmol) was added and the mixture stirred at rt under an atmosphere of nitrogen. After 16 h t.l.c. (petrol:ethyl acetate 1:1) indicated formation of a major product (Rf 0.75) and consumption of starting material (Rf 0.6). The reaction mixture was quenched by addition of sodium hydrogen carbonate (300 mL of a saturated solution), stirred for a further 2 h, separated and the organic layer dried (Na₂SO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate 2:1) to afford ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio- α -D-mannopyranoside **2.18** (22.0 g, 73%) as a white crystalline solid, m.p. 110-113 °C [lit. 107-108 °C];²³ [α]_D²⁰ + 87.1 (*c*, 1.0 in CHCl₃) [Lit. [α]_D²⁰ + 104 (*c*, 0.88 in CHCl₃)];²³ δ_{H} (500 MHz, CDCl₃) 1.29 (3H, t, *J* 7.5 Hz, SCH₂CH₃), 1.99, 2.04, 2.10, 2.17 (12H, 4 x s, 4 x CH₃), 2.60-2.66 (2H, m, SCH₂CH₃), 4.10 (1H, dd, *J*_{5,6} 2.0 Hz, *J*_{6,6'} 12.0 Hz, H-6), 4.32 (1H, dd, *J*_{5,6'} 5.0 Hz, H-6'), 4.40 (1H, m, H-5), 5.25-5.33 (4H, m, H-1, H-2, H-3, H-4).

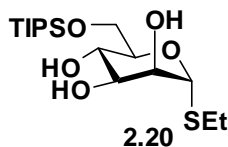
Ethyl 1-thio- α -D-mannopyranoside **2.19**



To a solution of Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio- α -D-mannopyranoside **2.18** (5.00 g, 12.7 mmol) in MeOH (250 mL) NaOMe (3.44 g, 63.7 mmol) was added in portions and the mixture stirred at rt under an atmosphere of nitrogen. After 16 h t.l.c. (methanol:ethyl acetate, 1:9) indicated formation of a single product (Rf 0.20) and complete consumption

of starting material (Rf 0.70). Dowex was added and stirred until the solution became neutral. The solution was filtered and concentrated *in vacuo* to afford ethyl 1-thio- α -D-mannopyranoside **4.3** (2.89 g, 100%) as a golden foam $[\alpha]_D^{20} + 199$ (c, 1.0 in MeOH); [lit. $[\alpha]_D^{18} + 228$ (c, 0.6 in CHCl_3)]²³. δ_H (500 MHz, CD_3OD) 1.12 (3H, t, J 10 Hz, SCH_2CH_3), 2.40-2.46 (2H, m, SCH_2CH_3), 3.45-3.47 (2H, m, H-3, H-4), 3.55 (1H, dd, $J_{5,6}$ 5.6 Hz, $J_{6,6'}$ 11.6 Hz, H-6), 3.64 (1H, dd, $J_{5,6'}$ 2.3 Hz, H-6'), 3.69-3.71 (2H, m, H-2, H-5), 5.08 (1H, s, H-1).

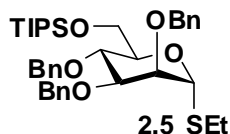
Ethyl 6-*O*-tri-*iso*-propylsilyl-1-thio- α -D-mannopyranoside **2.20**



Ethyl 1-thio- α -D-mannopyranoside **2.19** (2.0 g, 8.90 mmol) and imidazole (1.52 g, 22.3 mmol) were dissolved in THF (20 mL). The mixture was cooled to 0 °C and chloro-tri-*iso*-propylsilane (3.8 mL, 17.8 mmol) added. The reaction mixture was warmed to rt and stirred under an atmosphere of nitrogen. After 24 h t.l.c. (petrol:ethylacetate, 1:1) indicated formation of a major product (Rf 0.5) and consumption of starting material (Rf 0). The reaction mixture was diluted with ethyl acetate (50 mL), washed with ammonium chloride (2 x 25 mL of a saturated aqueous solution), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by flash column chromatography (ethyl acetate:petrol, 2:1) to afford ethyl 6-*O*-tri-*iso*-propylsilyl-1-thio- α -D-mannopyranoside **2.20** (3.0 g, 88.5%) as a white foam. $[\alpha]_D^{20} + 136$ (c, 1.0 in CHCl_3); [lit. $[\alpha]_D^{22} + 126$ (c, 0.6 in CHCl_3)]²³; δ_H (500 MHz, CDCl_3) 1.07-1.14 (21H, m, $\text{CH}(\text{CH}_3)_2$), 1.26 (3H, t, J 7.5 Hz, SCH_2CH_3), 2.51- 2.59 (2H, m, SCH_2CH_3), 2.88 (1H, d, J 4.0 Hz, OH), 3.13 (1H, br s,

OH), 3.66 (1H, br s, OH), 3.82-3.88 (2H, m, H-3, H-4), 3.94-3.97 (2H, m, H-6, H-6'), 4.02-4.06 (2H, H-2, H-5), 5.29 (1H, s, H-1).

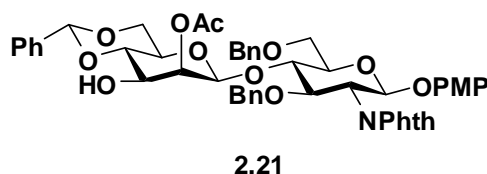
Ethyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl-1-thio- α -D-manno pyranoside **2.5**



Ethyl 6-*O*-tri-*iso*-propylsilyl-1-thio- α -D-mannopyranoside **2.20** (3.0 g, 7.88 mmol) was dissolved in THF (30 mL) and sodium hydride (1.89 g, 39.4 mmol of a 50% dispersion in mineral oil) added in portions. The mixture was stirred under an atmosphere of nitrogen for 10 min and then benzyl bromide (5.66 mL, 47.3 mmol) added in portions. The reaction mixture was heated to 60 °C and stirred for a further 20 h, after which time t.l.c. (petrol:ethyl acetate, 9:1) indicated formation of a major product (R_f 0.6) and consumption of starting material (R_f 0.0). Methanol (30 mL) was added and the solution stirred for 15 min then concentrated *in vacuo*. The residue was dissolved in ethylacetate (50 mL) and washed with water (2 x 30 mL). The aqueous washings were re-extracted with DCM (2 x 30 mL), the organics combined, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 19:1) to afford ethyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl-1-thio- α -D-mannopyranoside **2.5** (4.0 g, 78%) as a pale yellow oil, $[\alpha]_D^{20} + 71$ (c , 1.0 in CHCl_3); [lit. $[\alpha]_D^{22} + 66$ (c , 1.0 in CHCl_3)]²³; δ_H (500 MHz, CDCl_3) 1.0-1.17 (21H, m, $\text{CH}(\text{CH}_3)_2$), 1.23 (3H, t, J 7.4 Hz, SCH_2CH_3), 2.51-2.64 (2H, m, SCH_2CH_3), 3.81 (1H, brs., H-2), 3.87 (1H, dd, $J_{3,4}$ 8.65, 2.66 Hz, H-3), 3.91-4.02 (4H, m, H-4, H-5, H-6, H-6'),

4.57 , 4.62 (2H, m, PhCH₂), 4.63, 4.93 (1H, d, *J* 10.88 Hz PhCH₂), 4.67 (2H, br s, PhCH₂), 5.35 (1H, s, H-1), 7.24-7.42 (15H, m, 15 x Ar-H).

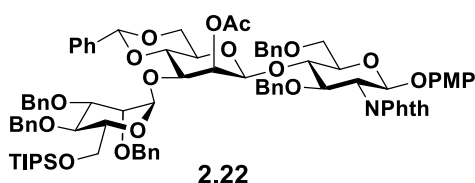
p*-Methoxyphenyl 2-*O*-acetyl-4,6-*O*-benzylidene-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.21*



Freshly distilled THF (15 mL) was added to a flame-dried flask containing (1,5-cyclooctadiene)bis(methyldiphenylphosphine) iridium(I) hexafluorophosphate (28 mg, 0.03 mmol). The mixture was degassed and subjected to an atmosphere of hydrogen for 15 min until the catalyst lost its pink colour and had fully dissolved. *p*-Methoxyphenyl 2-*O*-acetyl-3-*O*-allyl-4,6-*O*-benzylidene-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **2.4** (0.39 g, 0.42 mmol) was dissolved in freshly distilled THF (25 mL) in a second flame-dried flask. The solution of the activated Iridium catalyst was added and the mixture was stirred for 14 h under an atmosphere of nitrogen. After this time water (2.1 mL) and *N*-iodosuccinimide (0.281 g, 2.10 mmol) were added. After 18 h t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (*R_f* 0.20) and consumption of starting material (*R_f* 0.55). The reaction mixture was diluted with DCM (25 mL), washed with sodium thiosulfate (2 x 15 mL of a 10% solution), sodium hydrogen carbonate (2 x 15 mL of a saturated solution), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (1:1 petrol:ethyl acetate) to afford *p*-methoxyphenyl 2-*O*-acetyl-4,6-*O*-

benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6 di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.21** (0.280 g, 75% over two steps) as a white solid, m.p. 105-107°C [lit. 111-113];²³ $[\alpha]_{\text{D}}^{20} + 40$ (*c*, 1.0 in CHCl₃) [lit. $[\alpha]_{\text{D}}^{20} + 34.3$ (*c*, 0.75 in CHCl₃)];²³ δ H (500 MHz, CDCl₃) 2.19 (3H, s, CH₃), 2.23 (1H, d, $J_{\text{OH},3}$ 3.6 Hz, OH), 3.20 (1H, m, H-5b), 3.59 (1H, at, J 9.8 Hz, H-6b), 3.65-3.71 (2H, m, H-3b, H-5a), 3.72 (3H, s, OCH₃), 3.74-3.76 (1H, m, H-4b), 3.78 (1H, dd, $J_{5,6}$ 1.7 Hz, $J_{6,6'}$ 11.3 Hz, H-6a), 3.87 (1H, dd, $J_{5,6'}$ 3.1 Hz, H-6'a), 4.18-4.22 (2H, m, H-4a, H-6'b), 4.30-4.34 (1H, m, H-3a), 4.40-4.45 (2H, m, H-2a, PhCH₂), 4.51, 4.79 (2H, m, PhCH₂), 4.76 (1H, d, $J_{1,2}$ 0.6 Hz, H-1b), 4.85 (1H, d, J 12.2 Hz, PhCH₂), 5.30 (1H, dd, $J_{2,3}$ 3.5 Hz, H-2b), 5.50 (1H, s, PhCH(O)), 5.61 (1H, d, $J_{1,2}$ 8.4 Hz, H-1a), 6.69-6.72 (2H, m, 2 x Ar-H), 6.79-6.83 (2H, m, 2 x Ar-H), 6.88-6.95 (3H, m, 3 x Ar-H), 7.02-7.04 (2H, m, 2 x Ar H), 7.33-7.40 (8H, m, 8 x Ar-H), 7.46-7.48 (2H, m, 2 x Ar-H), 7.68-7.80 (4H, m, 4 x Ar-H)

p-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.22**

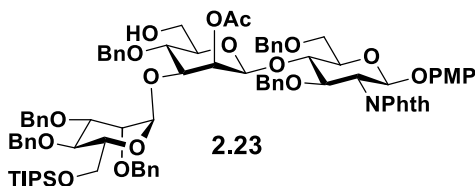


A solution of disaccharide **2.21** (1.00 g, 1.13 mmol), thioglycoside **2.5** (1.10 g, 1.69 mmol) and tri-*tert*-butylpyrimidine (1.26 g, 5.07 mmol) in DCM (40 mL) was added to a flame-dried round-bottom flask containing activated 3Å molecular sieves (3.0 g). The

solution was cooled to 0 °C under an atmosphere of nitrogen, stirred for 20 min and methyl trifluoromethanesulfonate (0.574 mL, 5.07 mmol) added. After 16 h t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.65) and complete consumption of the alcohol starting material **2.21** (R_f 0.25). Triethylamine (0.706 mL, 5.07 mmol) was added and the reaction mixture stirred for 10 min before being filtered through Celite[®] and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford trisaccharide **2.22** (1.52 g, 91%) as a white foam, $[\alpha]_D^{25} + 11.0$ (*c*, 0.5 in CHCl₃); ν_{\max} (KBr disk) 1751, 1716 (s, C=O) cm⁻¹; δ_H (500 MHz, CDCl₃) 1.06 (3H, s, CH(CH₃)₂), 1.07 (18H, s, CH(CH₃)₂), 2.03 (3H, s, CH₃), 3.11-3.15 (1H, m, H-5b), 3.56 (1H, at, *J* 10.4 Hz, H-6b), 3.64-3.70 (3H, m, H-2c, H-5a, H-5c), 3.71 (3H, s, OCH₃), 3.73 (1H, dd, *J*_{2,3} 2.6 Hz, *J*_{3,4} 9.9 Hz, H-3c), 3.77-3.81 (3H, m, H-4b, H-6a, H-6'a), 3.87 (1H, dd, *J*_{2,3} 3.5 Hz, *J*_{3,4} 9.9 Hz, H-3b), 3.93 (1H, d, *J* 9.9 Hz, H-6c), 3.98 (1H, dd, *J*_{5,6'} 4.4 Hz, *J*_{6,6'} 11.1 Hz, H-6'c), 4.04 (1H, at, *J* 9.5 Hz, H-4c), 4.15-4.19 (2H, m, H-4a, H-6'b), 4.31 (1H, dd, *J*_{2,3} 10.6 Hz, *J*_{3,4} 8.5 Hz, H-3a), 4.37-4.46 (4H, m, H-2a, 3 x PhCH₂), 4.53, 4.74 (2H, m, PhCH₂), 4.55, 4.61 (2H, m, PhCH₂), 4.68, 4.91 (2H, m, PhCH₂), 4.76 (1H, s, H-1b), 4.84 (1H, d, *J* 12.2 Hz, PhCH₂), 5.24 (1H, s, H-1c), 5.40 (1H, d, *J* 3.5 Hz, H-2b), 5.46 (1H, s, PhCH(O)), 5.59 (1H, d, *J*_{1,2} 8.4 Hz, H-1a), 6.68-6.70 (2H, m, 2 x Ar-H), 6.80-6.81 (2H, m, 2 x Ar-H), 6.90-6.96 (3H, m, 3 x Ar-H), 7.01-7.02 (2H, m, 2 x Ar-H), 7.09-7.11 (2H, m, 2 x Ar-H), 7.14-7.19 (3H, m, 3 x Ar-H), 7.25-7.38 (18H, m, 18 x Ar-H), 7.43-7.45 (2H, m, 2 x Ar-H), 7.67-7.73 (4H, m, 4 x Ar-H); δ_C (125.8 MHz, CDCl₃) 12.2 (d, CH(CH₃)₂), 18.2 (q, CH(CH₃)₂), 21.0 (s, CH₃), 55.7 (q, OCH₃), 55.8 (d, C-2a), 63.2 (t, C-6c), 66.6 (d, C-5b), 68.3 (t, C-6a), 68.7 (t, C-6b), 71.1 (d, C-2b), 71.9, 72.2, 73.6, 74.7, 74.9 (5 x t, 5 x PhCH₂), 73.2 (d, C-3b), 74.1

(d, C-5c), 74.5 (d, C-4c), 74.9 (d, C-5a), 75.4 (d, C-2c), 77.2 (d, C-3a), 78.6 (d, C-4a), 79.1 (d, C-4b), 79.4 (d, C-3c), 97.8 (d, C-1a), 98.7 (d, C-1c), 99.3 (d, C-1b), 101.9 (d, PhCH(O)), 114.5, 118.8, 126.2, 127.3, 127.4, 127.4, 127.5, 127.7, 127.8, 127.9, 128.0, 128.0, 128.0, 128.1, 128.2, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.7, 129.4, 134.0, (24 x d, 38 x Ar-C), 137.4, 138.1, 138.6, 138.6, 138.9, 139.2, 151.0, 155.5 (10 x s, 10 x Ar-C), 169.6 (s, C=O); HRMS (ES⁺) Calculated For C₈₆H₉₈NO₁₉Si (MH⁺) 1476.6502; Found (MH⁺):1476.6505; C₈₆H₉₇NNaO₁₉Si (MNa⁺) 1498.6322. Found (MNa⁺):1498.6325.

p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.23*

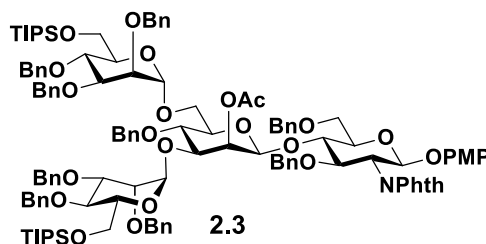


A solution of *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.22** (0.150 g, 0.102 mmol) in DCM (5 mL) was added to a flame-dried round-bottom flask containing activated 3Å molecular sieves (0.30 g). The solution was stirred at rt under an atmosphere of nitrogen for 30 min then cooled to -78 °C. Triethylsilane (49 μ L, 0.31 mmol) and dichlorophenylborane (45 μ L, 0.35 mmol) were added and the reaction mixture stirred at

-78 °C. After 45 min t.l.c. (petrol:ethyl acetate, 3:2) indicated formation of a single product (R_f 0.30) and consumption of starting material (R_f 0.50). Triethylamine (0.75 mL) and methanol (0.75 mL) were added, the reaction mixture diluted with DCM (10.0 mL), washed with sodium hydrogen carbonate (2 x 5 mL of a saturated solution), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was co-distilled with methanol five times at 50 °C before being purified by flash column chromatography (3:2 petrol:ethyl acetate) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.23** (0.143 g, 94%) as a white foam, $[\alpha]_D^{25} + 27$ (*c*, 0.5 in $CHCl_3$); ν_{max} (KBr disk) 3445 (s, O-H), 1749, 1716, 1636 (s, C=O) cm^{-1} ; δ_H (500 MHz, $CDCl_3$) 1.07 (3H, s, $CH(CH_3)_2$), 1.08 (18H, s, $CH(CH_3)_2$), 2.07 (3H, s, CH_3), 3.07-3.09 (1H, m, H-5b), 3.42-3.44 (1H, m, H-6b), 3.58-3.62 (3H, m, H-3b, H-4b, H-5c), 3.65-3.68 (3H, m, H-2c, H-5a, H-6'b), 3.71 (3H, s, OCH_3), 3.77 (1H, dd, $J_{5,6}$ 2.9 Hz, $J_{6,6'}$ 9.5 Hz, H-6a), 3.80-3.82 (2H, m, H-3c, H-6'a), 3.92 (1H, dd, $J_{5,6}$ 1.1 Hz, $J_{6,6'}$ 11.3 Hz, H-6c), 4.03 (1H, dd, $J_{5,6'}$ 3.2 Hz, H-6'c), 4.15 (1H, at, J 9.4 Hz, H-4a), 4.19 (1H, at, J 9.6 Hz, H-4c), 4.29 (1H, dd, $J_{2,3}$ 10.6 Hz, $J_{3,4}$ 9.7 Hz, H-3a), 4.37-4.42 (3H, m, H-2a, 2 x $PhCH_2$), 4.48-4.57 (5H, m, 5 x $PhCH_2$), 4.64 (1H, d, J 12.0 Hz, $PhCH_2$), 4.68 (1H, s, H-1b), 4.72-4.75 (2H, m, 2 x $PhCH_2$), 4.88 (2H, at, J 10.9 Hz, 2 x $PhCH_2$), 5.07 (1H, d, $J_{1,2}$ 1.7 Hz, H-1c), 5.35 (1H, d, J 2.4 Hz, H-2b), 5.60 (1H, d, $J_{1,2}$ 8.5 Hz, H-1a), 6.69-6.71 (2H, m, 2 x Ar-H), 6.79-6.81 (2H, m, 2 x Ar-H), 6.92-6.98 (3H, m, 3 x Ar-H), 7.01-7.03 (2H, m, 2 x Ar-H), 7.20-7.34 (25H, m, 25 x Ar-H), 7.67-7.79 (4H, m, 4 x Ar-H); δ_C (125.8 MHz, $CDCl_3$) 12.2 (d, $CH(CH_3)_2$), 18.2 (q, $CH(CH_3)_2$), 21.2 (s, CH_3), 55.7 (m, C-2a, OCH_3), 61.8 (t, C-6b), 62.6 (t, C-6c), 68.3 (t,

C-6a), 71.3 (d, C-2b), 72.4, 72.5, 73.7, 74.6, 74.7, 74.9 (6 x t, 6 x PhCH₂), 74.2 (d, C-5c), 74.3 (d, C-4c), 74.7 (d, C-4b), 74.9 (d, C-5a), 75.4 (d, C-5b), 76.5 (d, C-2c), 77.0 (d, C-3a), 78.3 (d, C-4a), 78.3 (d, C-3b), 79.6 (d, C-3c), 97.7 (d, C-1b), 98.6 (d, C-1c), 100.7 (d, C-1a), 114.5, 118.8, 127.3, 127.3, 127.4, 127.5, 127.5, 127.5, 127.6, 127.7, 128.0, 128.0, 128.2, 128.2, 128.4, 128.4, 128.4, 128.6, 128.7, 133.7, (20 x d, 38 x Ar-C), 138.0, 138.1, 138.4, 138.7, 138.9, 139.2, 150.9, 155.5 (8 x s, 10 x Ar-C), 169.7 (s, C=O); HRMS (ES⁺) Calculated For C₈₆H₉₉NNaO₁₉Si (MNa⁺) 1500.6478. Found (MNa⁺):1500.6473.

***p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside 2.3**



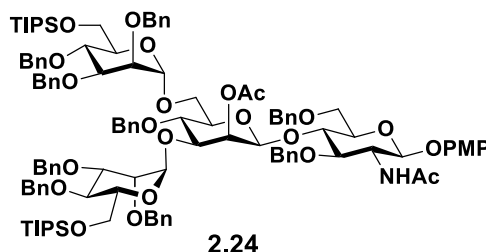
A solution of *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.23** (0.179 g, 0.121 mmol), thioglycoside **2.5** (0.118 g, 0.182 mmol) and tri-*tert*-butylpyrimidine (0.135 g, 0.545 mmol) in DCM (8 mL) was added to a flame-dried round-bottom flask containing

activated 3Å molecular sieves (0.3 g). The solution was cooled to 0 °C under an atmosphere of nitrogen, stirred for 20 min and methyl trifluoromethanesulfonate (62 µL, 0.55 mmol) added. After 16 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of a major product (R_f 0.55) and complete consumption of the alcohol starting material **2.23** (R_f 0.20). Triethylamine (76 µL, 0.55 mmol) was added and the reaction mixture stirred for 10 min before being filtered through celite and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1→3)-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1→6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.3** (0.198 g, 79%) as a white foam, $[\alpha]_D^{25} + 24$ (c, 0.25 in CHCl₃); v_{\max} (KBr disk) 1717, 1630 (s, C=O) cm⁻¹; δ_H (500 MHz, CDCl₃) 1.00, 1.03 (6H, 2 x s, 2 x CH(CH₃)₂), 1.11, 1.12 (36H, 2 x s, 2 x CH(CH₃)₂), 2.06 (3H, s, CH₃), 3.11-3.22 (1H, m, H-5b), 3.59-3.76 (6H, m, H-3b, H-5c, H-5d, H-6a, H-6'a, H-6b), 3.72 (3H, s, OCH₃), 3.78-3.89 (6H, m, H-2c, H-2d, H-4b, H-5a, H-6'b, H-6c), 3.94-4.00 (3H, m, H-3a, H-3d, H-6'c), 4.03-4.09 (2H, m, H-3c, H-6d), 4.14-4.29 (4H, m, H-4a, H-4c, H-4d, H-6'd), 4.35-4.70 (14H, m, H-2a, 13 x PhCH₂), 4.73-4.78 (3H, m, H-1b, 2 x PhCH₂), 4.83-4.95 (4H, m, H-1d, 3 x PhCH₂), 5.08 (1H, s, H-1c), 5.39 (1H, s, H-2b), 5.58 (1H, d, $J_{1,2}$ 8.4 Hz, H-1a), 6.68-6.76 (4H, m, 4 x Ar-H), 6.78-6.82 (4H, m, 4 x Ar-H), 6.93-6.98 (2H, m, 2 x Ar-H), 7.17-7.37 (39H, m, 39 x Ar-H), 7.62-7.67 (4H, m, 4 x Ar-H); δ_C (125.8 MHz, CDCl₃) 12.1, 12.2 (2 x d, 2 x CH(CH₃)₂), 18.0, 18.2 (2 x q, 2 x CH(CH₃)₂), 21.2 (s, CH₃), 55.7 (m, C-2a, OCH₃), 62.7 (t, C-6c), 62.9 (t, C-6d), 66.2 (t, C-6b), 68.3 (t, C-6a), 71.6 (d, C-2b), 71.6, 72.4, 72.5, 72.8, 74.4, 74.4, 74.5, 74.8, 74.9 (9 x t, 9 x PhCH₂), 73.4 (d,

C-5c), 73.6 (d, C-4d), 73.9 (d, C-5b), 74.2 (d, C-4a), 74.4 (d, C-4c), 74.6 (d, C-5d), 74.9 (d, C-4b), 75.3 (d, C-5a), 75.5 (d, C-2d), 76.3 (d, C-2c), 77.2 (d, C-3a), 78.4 (d, C-3b), 79.2 (d, C-3d), 79.8 (d, C-3c), 97.8 (d, C-1a), 98.6 (d, C-1d), 99.6 (d, C-1b), 100.6 (d, C-1c), 114.4, 118.6, 126.6, 127.0, 127.1, 127.2, 127.4, 127.5, 127.6, 127.7, 127.7, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.2, 128.2, 128.3, 128.4, 128.5, 128.6, 128.6, 129.6, 129.6, 132.8, 133.4, 135.8, 135.8, 136.1 (32 x d, 53 x Ar-C), 133.7, 133.7, 134.0, 134.1, 138.0, 138.1, 138.6, 138.6, 138.8, 138.9, 139.2, 151.2, 155.5 (13 x s, 13 x Ar-C), 169.5, 169.6, 171.0 (3 x s, 3 x C=O); HRMS (ES^+) Calculated For $\text{C}_{122}\text{H}_{147}\text{NNaO}_{24}\text{Si}_2$ (MNa^+) 2089.9783. Found (MNa^+):2089.9838.

The stereochemistry of the anomeric linkages was confirmed by obtaining the $\text{H}^1\text{-C}^{13}$ coupling constants from C-coupled HSQC experiments. They were as follows: C(1a)-H(1a) 168 Hz; C(1b)-H(1b) 162 Hz; C(1c)-H(1c) 175 Hz; C(1d)-H(1d) 175 Hz.

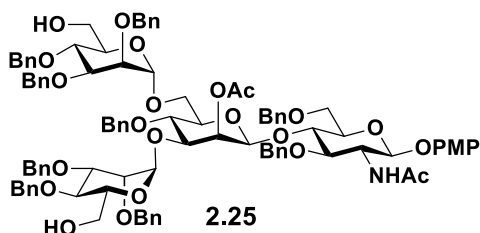
***p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl - (1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside 2.24**



p-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.3** (0.120 g, 0.0580 mmol) was dissolved in methanol (6 mL) and ethylene diamine (3 mL) and the solution heated to reflux under an atmosphere of nitrogen. After 16 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of a single product (R_f 0.25) and complete consumption of starting material (R_f 0.40). The reaction mixture was concentrated *in vacuo* and co-distilled five times with toluene. The residue was dissolved in pyridine (3 mL), cooled to 0 °C and acetic anhydride (1 mL) added. The reaction mixture was stirred at rt under an atmosphere of argon. After 20 h t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of a single product (R_f 0.40) and complete consumption of amine intermediate (R_f 0.25). The reaction mixture was concentrated *in vacuo*, the residue dissolved in DCM (10 mL), washed with water (10 mL), sodium hydrogen carbonate (2 x 10 mL of a saturated aqueous solution), dried (MgSO₄) and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 3:2) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl -(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside **2.24** (0.104 g, 91%) as a pale yellow foam, $[\alpha]_D^{25} + 35$ (c, 0.18 in CHCl₃); ν_{\max} (KBr disk) 3441 (s, N-H stretch), 1751, 1635 (s, C=O) cm⁻¹; δ_H (500 MHz, CDCl₃) 1.02 (6H, m, 2 x CH(CH₃)₂), 1.05, 1.06 (36H, 2 x s, CH(CH₃)₂), 1.61, 1.94 (6H, 2 x s, 2 x CH₃), 3.29-3.30 (1H, m, H-5b), 3.40-3.46

(2H, m, H-6b, H-6c), 3.56-3.79 (13H, m, H-2a, H-2c, H-2d, H-3b, H-3d, H-4b, H-5a, H-5c, H-5d, H-6a, H-6'a, H-6'b, H-6'c), 3.76 (3H, s, OCH₃), 3.86-3.90 (2H, m, H-3c, H-6d), 3.94 (1H, dd, $J_{5,6'}$ 3.6 Hz, $J_{6,6'}$ 11.0 Hz, H-6'd), 4.04 (1H, at, J 7.9 Hz, H-3a), 4.07-4.16 (3H, m, H-4a, H-4c, H-4d), 4.37 (1H, d, J 12.4 Hz, PhCH₂), 4.40 (1H, d, J 11.7 Hz, PhCH₂), 4.42 (1H, d, J 11.9 Hz, PhCH₂), 4.46-4.59 (10H, m, 10 x PhCH₂), 4.61 (1H, d, J 11.7 Hz, PhCH₂), 4.66 (1H, d, J 12.0 Hz, PhCH₂), 4.70 (1H, s, H-1b), 4.76 (1H, d, J 11.6 Hz, PhCH₂), 4.81 (1H, s, H-1d), 4.87 (1H, d, J 11.2 Hz, PhCH₂), 4.88 (1H, d, J 10.9 Hz, PhCH₂), 5.09 (1H, d, $J_{1,2}$ 1.3 Hz, H-1c), 5.27 (1H, d, $J_{2,NH}$ 7.5 Hz, NH), 5.33 (1H, d, $J_{1,2}$ 6.9 Hz, H-1a), 5.37 (1H, d, J 3.0 Hz, H-2b), 6.76-6.79 (2H, m, 2 x Ar-H), 6.88-6.90 (2H, m, 2 x Ar-H), 7.16-7.32 (45H, m, 45 x Ar-H); δ_C (125.8 MHz, CDCl₃) 12.2, 12.2 (2 x d, 2 x CH(CH₃)₂), 18.1, 18.2 (2 x q, 2 x CH(CH₃)₂), 21.0, 23.3 (2 x s, 2 x CH₃), 55.4 (d, C-2a), 55.8 (q, OCH₃), 62.5 (t, C-6c), 62.8 (t, C-6d), 65.9 (t, C-6b), 69.3 (t, C-6a), 71.4 (d, C-2b), 72.3, 72.4, 72.4, 72.6, 73.5, 74.3, 74.7, 74.8, 75.6 (9 x t, 9 x PhCH₂), 73.3 (d, C-5c), 73.9 (d, C-5a), 74.1 (d, C-5d), 74.3 (d, C-4d), 74.6 (d, C-5b), 75.1 (d, C-4a), 75.2 (d, C-4c), 75.4 (d, C-4b), 75.8 (d, C-2d), 76.1 (d, C-2c), 77.3 (d, C-3a), 79.0 (d, C-3b), 79.6 (d, C-3d), 80.6 (d, C-3c), 97.4 (d, C-1b), 98.1 (d, C-1d), 98.8 (d, C-1a), 100.4 (d, C-1c), 114.4, 118.6, 126.6, 127.0, 127.1, 127.2, 127.4, 127.5, 127.6, 127.7, 127.7, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.2, 128.2, 128.3, 128.4, 128.5, 128.6, 128.6, 129.6, 129.6, 132.8, 133.4 (29 x d, 49 x Ar-C), 138.0, 138.3, 138.6, 138.7, 138.8, 138.9, 139.1, 139.1, 139.4, 151.6, 155.2 (11 x s, 11 x Ar-C), 169.8, 170.5 (2 x s, 2 x C=O); HRMS (ES⁺) Calculated For C₁₁₆H₁₄₈NO₂₃Si₂ (MH⁺) 1980.0014. Found (MH⁺):1980.0041.

p-Methoxyphenyl 2,3,4-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl (1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside **2.25**



p-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl - (1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside **2.24** (140 mg, 0.07 mmol) was dissolved in anhydrous DCM (5 mL) under an atmosphere of nitrogen in a flame-dried flask. The solution was cooled to 0 °C and boron trifluoride diethyl etherate (43 μ L, 0.35 mmol) was added dropwise. The reaction was stirred at 0 °C for 1 h after which time t.l.c. (petrol: ethyl acetate, 1:1) indicated complete consumption of starting material (R_f 0.55) and formation of a single product (R_f 0.2). The reaction mixture was diluted with DCM (10 mL), washed with sodium hydrogen carbonate (10 mL of a saturated aqueous solution), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol: ethyl acetate, 1:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl (1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside **2.25** (88 mg, 76%) as a white foam. $[\alpha]_D^{20} +32$ (*c*, 1.0 in

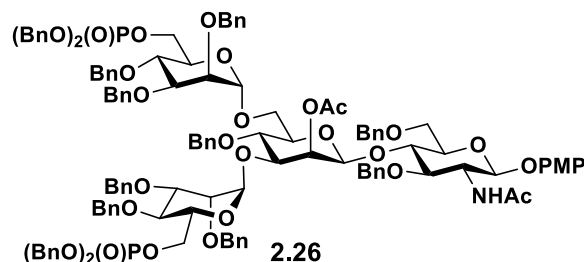
CHCl₃); ν_{\max} (KBr disk) 3428 (s, N-H stretch), 1746, 1739 (s, C=O) cm⁻¹; δ_{H} (500 MHz, CDCl₃) 1.58, 1.97 (6H, 2 x s, 2 x CH₃), 3.32-3.38 (1H, m, H-5b), 3.40-3.48 (2H, m, H-6b, H-6c), 3.56-3.79 (13H, m, H-2a, H-2c, H-2d, H-3b, H-3d, H-4b, H-5a, H-5c, H-5d, H-6a, H-6'a, H-6'b, H-6'c), 3.76 (3H, s, OCH₃), 3.82-4.0 (4H, m, H-3c, H-6d, H-6'd, H-3a), 4.13-4.20 (3H, m, H-4a, H-4c, H-4d), 4.43-4.51 (3H, m, PhCH₂) 4.52-4.61 (10H, m, 10 x PhCH₂), 4.63-4.64 (2H, m, PhCH₂), 4.70 (1H, s, H-1b), 4.95 (1H, s, H-1d), 5.10 (1H, s, H-1c), 5.33 (1H, d, $J_{1,2}$ 6.4 Hz, H-1a), 5.37 (1H, d, $J_{2,3}$ 3.0 Hz, H-2b), 6.77-6.79 (2H, m, 2 x Ar-H), 6.88-6.90 (2H, m, 2 x Ar-H), 7.19-7.32 (45H, m, 45 x Ar-H); δ_{C} (125 MHz, CDCl₃) 21.0, 23.3 (2 x s, 2 x CH₃), 55.64 (q, OCH₃), 56.62 (d, C-2a), 61.9 (t, C-6c), 62.3 (t, C-6d), 66.4 (t, C-6b), 68.7 (t, C-6a), 71.2 (d, C-2b), 72.2, 72.3, 72.6, 72.7, 73.5, 74.2, 74.6, 74.7, 75.4 (9 x t, 9 x PhCH₂), 73.4 (d, C-5c), 74.1 (d, C-5a), 74.5 (d, C-5d), 75.0 (d, C-4d), 75.2 (d, C-5b), 75.4 (d, C-4a), 75.5 (d, 4b), 75.6 (d, C-4c), 75.8 (d, C-2d), 76.0 (d, C-2c), 77.3 (d, C-3a), 79.6 (d, C-3b), 79.7 (d, C-3d), 80.3 (d, C-3c), 97.5 (d, C-1b), 98.1 (d, C-1d), 98.7 (d, C-1a), 100.9 (d, C-1c), 114.4, 118.6, 126.6, 127.0, 127.1, 127.2, 127.4, 127.5, 127.6, 127.7, 127.7, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.2, 128.2, 128.3, 128.4, 128.5, 128.6, 128.6, 129.6, 129.6, 132.8, 133.4 (29 x d, 49 x Ar-C), 138.0, 138.3, 138.6, 138.7, 138.8, 138.9, 139.1, 139.1, 139.4, 151.6, 155.2 (11 x s, 11 x Ar-C), 170.0, 170.4 (2 x s, 2 x C=O); HRMS (ES⁺) Calculated For C₉₈H₁₀₈NO₂₃ (M+H) 1667.7346. Found (M+H): 1667.7350.

***p*-Methoxyphenyl**

2,3,4-tri-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl- α -D-

mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl- α -D

mannopyranosyl-(1→6)]-2-*O*-acetyl-4-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside 2.26



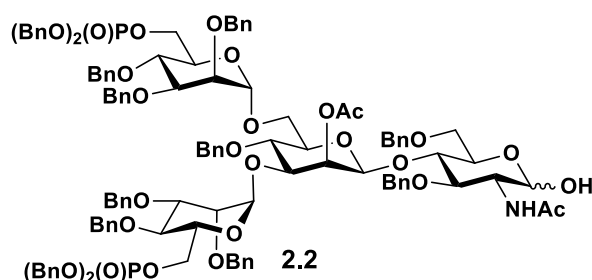
Dibenzyl N,N-diisopropyl phosphoramidate (0.053 mL, 0.16 mmol) and 1*H*-tetrazole (0.48 mL of a 0.45 M solution in acetonitrile) were added to a flame-dried flask and dissolved in DCM (0.45 mL). The solution was stirred at rt 10 min under nitrogen atmosphere. *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[2,3,4-tri-*O*-benzyl-6--α-D-mannopyranosyl-(1→6)]-2-*O*-acetyl-4-*O*-benzyl-β-D-mannopyranosyl (1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside **2.25** (0.04 mg, 0.02 mmol) in DCM (0.45 mL) was added, and the reaction stirred for 18 h. After this time t.l.c. (petrol:ethyl acetate, 1:1) indicated complete consumption of alcohol starting material (R_f 0.30) and formation of a major product (R_f 0.60). The reaction mixture was cooled to -78°C and *m*-chloroperbenzoic acid (0.035 g, 0.2 mmol) was added. The mixture was stirred for 2 h and then warmed to rt, after which time t.l.c. (petrol:ethyl acetate, 1:1) indicated complete consumption of the phosphine intermediate (R_f 0.60) and formation of a major product (R_f 0.25). The reaction was quenched by addition of sodium bisulfite (5 mL of a 10% w/v aqueous solution), stirred for 10 min, and extracted with DCM (10 mL). The organic extracts were washed with sodium hydrogen carbonate (5 mL of a saturated aqueous solution) and brine (5 mL), dried (Na₂SO₄), filtered and

concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 1:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside

2.26 (0.25 g, 58% over two steps) as a white foam. $[\alpha]_D^{20} +41$ (*c*, 1.0 in CHCl₃); δ_H (400 MHz, CDCl₃) 1.61, 1.89 (6H, 2 x s, 2 x CH₃), 3.29-3.30 (1H, m, H-5b), 3.42-3.48 (2H, m, H-6b, H-6c), 3.56-3.79 (13H, m, H-2a, H-2c, H-2d, H-3b, H-3d, H-4b, H-5a, H-5c, H-5d, H-6a, H-6'a, H-6'b, H-6'c), 3.76 (3H, s, OCH₃), 3.86-3.94 (3H, m, H-3c, H-6d, H-6'd), 4.04-4.16 (4H, m, H-3a, H-4a, H-4c, H-4d), 4.37-4.42 (3H, m, PhCH₂), 4.46-4.59 (10H, m, 10 x PhCH₂), 4.61-4.66 (2H, m, PhCH₂), 4.70 (1H, s, H-1b), 4.76 (1H, d, 10.6 Hz, PhCH₂), 4.81 (1H, s, H-1d), 4.87 (1H, d, 8 Hz, PhCH₂), 4.88 (1H, d, 10.2 Hz, PhCH₂), 5.10 (1H, s, H-1c), 5.21 (2H, m, PhCH₂), 5.33 (1H, d, $J_{1,2}$ 6.4 Hz, H-1a), 5.37 (1H, d, $J_{2,3}$ 3.0 Hz, H-2b), 6.76-6.79 (2H, m, 2 x Ar-H), 6.88-6.90 (2H, m, 2 x Ar-H), 7.16-7.45 (65H, m, 65 x Ar-H); δ_C (100 MHz, CDCl₃) 21.0, 23.3 (2 x CH₃), 55.64 (OCH₃), 56.62 (C-2a), 66.0 (C-6c), 66.8 (C-6d), 67.1 (C-6b), 67.8 (C-6a), 71.2 (C-2b), 72.2, 72.3, 72.4, 72.5, 72.6, 72.7, 73.5, 74.0, 74.3, 74.6, 74.7, 75.0, 75.6 (13 x PhCH₂), 73.2 (C-5c), 74.1 (C-5a), 74.5 (C-5d), 75.1 (C-4d), 75.2 (C-5b), 75.4 (C-4a), 75.5 (4b), 75.7 (C-4c), 75.8 (C-2d), 76.2 (C-2c), 77.3 (C-3a), 78.1 (C-3b), 79.7 (C-3d), 80.7 (C-3c), 96.9 (C-1b), 97.9 (C-1d), 98.4 (C-1a), 100.2 (C-1c), 114.4, 118.4, 126.6, 126.9, 127.1, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.6, 127.6, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 128.0, 128.1, 128.2, 128.2, 128.3, 128.3, 128.3, 128.3, 128.3, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.7, 130.1, 133.1, 136.0 (41 x d, 69 x Ar-C)

135.8, 135.9, 136.0, 136.2, 137.7, 138.0, 138.1, 138.3, 138.4, 138.4, 138.5, 138.7, 139.0, 151.4, 155.1 (15 x Ar-C), 170.0, 170.5 (2 x s, 2 x C=O); δ_p (162 MHz, $CDCl_3$) – 1.02, – 1.20; HRMS (ES^+) Calculated For $C_{126}H_{134}NO_{29}P_2$ (MH^+) 2187.8550. Found (MH^+): 2187.8561.

2,3,4-tri-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranose 2.2

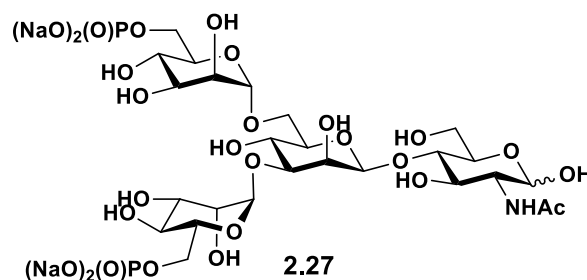


p-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl- α -D mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy- β -D-glucopyranoside **2.26** (15 mg, 0.01 mmol) was suspended in a mixture of acetonitrile (0.8 mL) and water (0.21 mL). Ceric ammonium nitrate (27 mg, 0.05 mmol) was added, and the reaction mixture stirred at rt. After 1 h, t.l.c. (ethyl acetate) indicated complete consumption of starting material (R_f 0.25) and formation of a single product (R_f 0.15). The reaction mixture was diluted with DCM (10 mL) and washed with sodium hydrogen carbonate (2 x 5 mL of a saturated aqueous solution), sodium thiosulfate (2 x 5

mL of a 5% w/v aqueous solution), EDTA (2 x 5 mL of a 0.1 M aqueous solution) and water (5 mL). The organic extracts were dried (Na₂SO₄), filtered, concentrated *in vacuo*, and the residue purified by flash column chromatography (ethyl acetate) to afford 2,3,4-tri-*O*-benzyl-6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranose **2.2** (11mg, 78%) as a pale yellow foam. δ_{H} (400 MHz, CDCl₃) 1.61, 1.89 (6H, 2 x s, 2 x CH₃), 3.29-3.30 (1H, m, H-5b), 3.42-3.48 (2H, m, H-6b, H-6c), 3.56-3.79 (13H, m, H-2a, H-2c, H-2d, H-3b, H-3d, H-4b, H-5a, H-5c, H-5d, H-6a, H-6'a, H-6'b, H-6'c), 3.76 (3H, s, OCH₃), 3.86-3.94 (3H, m, H-3c, H-6d, H-6'd), 4.04-4.16 (4H, m, H-3a, H-4a, H-4c, H-4d), 4.37-4.42 (3H, m, PhCH₂) 4.46-4.59 (10H, m, 10 x PhCH₂), 4.61-4.66 (2H, m, PhCH₂), 4.70 (1H, s, H-1b), 4.76 (1H, d, 10.4 Hz, PhCH₂), 4.81 (1H, s, H-1d), 4.87 (1H, d, 11 Hz, PhCH₂), 4.88 (1H, d, 10.2 Hz, PhCH₂), 5.10 (1H, s, H-1c), 5.21 (2H, m, PhCH₂) 5.33 (1H, d, $J_{1,2}$ 6.4 Hz, H-1a), 5.37 (1H, d, $J_{2,3}$ 3.0 Hz, H-2b), 7.16-7.45 (65H, m, 65 x Ar-H); δ_{C} (100 MHz, CDCl₃) 21.0, 23.3 (2 x CH₃), 56.62 (C-2a), 66.0 (C-6c), 66.8 (C-6d), 67.1 (C-6b), 67.8 (C-6a), 71.2 (C-2b), 72.2, 72.3, 72.4, 72.5, 72.6, 72.7, 73.5, 74.0, 74.3, 74.6, 74.7, 75.0, 75.6 (13 x PhCH₂), 73.2 (C-5c), 74.1 (C-5a), 74.5 (C-5d), 75.1 (C-4d), 75.2 (C-5b), 75.4 (C-4a), 75.5 (4b), 75.7 (C-4c), 75.8 (C-2d), 76.2 (C-2c), 77.3 (C-3a), 78.1 (C-3b), 79.7 (C-3d), 80.7 (C-3c), 96.9 (C-1b), 97.9 (C-1d), 98.4 (C-1a), 100.2 (C-1c), 126.6, 126.9, 127.1, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.6, 127.6, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 128.0, 128.1, 128.2, 128.2, 128.3, 128.3, 128.3, 128.3, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.7, 130.1, 133.1, 136.0 (39 x d, 65 x Ar-C) 135.8, 135.9, 136.0, 136.2, 137.7, 138.0, 138.1,

138.3, 138.4, 138.4, 138.5, 138.7, 139.0 (13 x s, 13 x Ar-C), 170.0, 170.5 (2 x s, 2 x C=O); δ_p (162 MHz, $CDCl_3$) – 1.04, – 1.10; HRMS (ES^+) Calculated For $C_{119}H_{128}NO_{28}P_2$ (MH^+) 2081.8132. Found (MH^+):2081.8191.

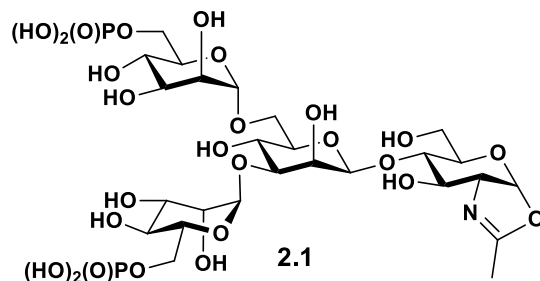
6-*O*-Phosphate- α -D-mannopyranosyl-(1 \rightarrow 3)-[6-*O*-phosphate- α -D-mannopyranosyl-(1 \rightarrow 6)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-2-deoxy-D-glucopyranose **2.27**



2,3,4-tri-*O*-benzyl-6-*O*-dibenzylphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 3)-[2,3,4-tri-*O*-benzyl-6-*O*-dibenzylphosphoryl- α -D-mannopyranosyl-(1 \rightarrow 6)]-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranose **2.2** (12 mg, 0.01 mmol) in THF (3 mL) was added to $NH_3(l)$ (20 mL) at – 33° C. The minimum amount of sodium for the mixture to turn deep blue was added to the stirred mixture. After 30 min MeOH (4 mL) was added, and reaction mixture was stirred for a further 1 h., warmed to rt, and the solvent was removed *in vacuo*. Gel filtration of the crude residue on a Sephadex G-10 column (eluting with 0.01% NH_3) afforded 6-*O*-Phosphate- α -D-mannopyranosyl-(1 \rightarrow 3)-[6-*O*-phosphate- α -D-mannopyranosyl-(1 \rightarrow 6)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-2-deoxy-D-glucopyranose **2.27** (4.9mg, 98%), as a white foam. δ_H (400 MHz, D_2O) 1.89 (3H, s, CH_3), 3.46-3.55 (1H, m, H-5b), 3.61-3.95 (16H, m, H-6b, H-6c, H-2a, H-2c, H-2d, H-3b,

H-3d, H-4b, H-5a, H-5c, H-5d, H-6a, H-6'a, H-6'b, H-6'c, H-3c, H-2b), 3.94-4.11 (2H, m, H-6d, H-6'd), 4.7-4.9 (4H, m, H-3a, H-4a, H-4c, H-4d), 5.0 (2H, m, H-1b, H-1c), 5.1 (1H, s, H-1a); δ_C (100 MHz, D₂O) 21.9 (CH₃), 46.6 (C-2a), 60.0 (C-6c), 62.8 (C-6d), 63.0 (C-6b), 63.4 (C-6a), 65.1 (C-2b), 65.5 (C-5c), 65.7 (C-5a), 66.0 (C-5d), 66.3 (C-4d), 68.0 (C-5b), 68.9 (C-4a), 71.7 (4b), 71.9 (C-4c), 72.6 (C-2d), 73.8 (C-2c), 74.5 (C-3a), 79.8 (C-3b), 80.2 (C-3d), 80.5 (C-3c), 99.5 (C-1b), 99.7 (C-1d), 100.2 (C-1a), 102.5 (C-1c), 174.5 (s, C=O); δ_P (162 MHz, D₂O) 4.70, 4.76; HRMS (ES⁺) Calculated For C₂₆H₄₈NO₂₇P₂ (MH⁺) 868.1889. Found (MH⁺): 868.1894.

2-Methyl [6-*O*-phosphate- α -D-mannopyranosyl-(1 \rightarrow 3)]-[6-*O*-phosphate- α -D-mannopyranosyl-(1 \rightarrow 6)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-1,2-deoxy-D-glucopyranose]-[2,1-*d*]-oxazoline 2.1

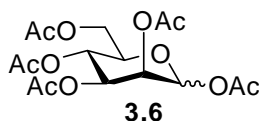


6-*O*-Phosphate- α -D-mannopyranosyl-(1 \rightarrow 3)-[6-*O*-phosphate- α -D-mannopyranosyl (1 \rightarrow 6)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-2-deoxy-D-glucopyranose **2.27** (2 mg, 0.0023 mmol) and triethylamine (3 μ L, 0.021 mmol) were dissolved in D₂O (18.4 μ L) and the resulting solution was cooled to 0°C. DMC (12 mg, 0.007 mmol) was added to the solution and the mixture was stirred for 15 min at the same temperature. Gel filtration of the residue on a Sephadex G-10 column eluted with 0.01% NH₃ afforded 2-Methyl [6-

O-phosphate- α -D-mannopyranosyl-(1 \rightarrow 3)-[6-*O*-phosphate- α -D-mannopyranosyl-(1 \rightarrow 6)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-1,2-deoxy-D-glucopyrano]-[2,1-*d*]-oxazoline **2.1** (1.8 mg, 95%) as a white foam. δ_{H} (400 MHz, D₂O) 1.89 (3H, s, CH₃), 2.8-2.95 (1H, m, H-5b), 3.26-3.95 (16H, m, H-6b, H-6c, H-2a, H-2c, H-2d, H-3b, H-3d, H-4b, H-5a, H-5c, H-5d, H-6a, H-6'a, H-6'b, H-6'c, H-3c, H-2b), 3.96-4.11 (2H, m, H-6d, H-6'd), 4.7-4.9 (6H, m, H-3a, H-4a, H-4c, H-4d, H-1b, H-1c), 5.95 (1H, d, *J* 7.3 Hz H-1a). δ_{P} (162 MHz, CDCl₃) 4.38; HRMS (ES⁺) Calculated For C₂₆H₄₆NO₂₆P₂ (MH⁺) 850.1783. Found (MH⁺):850.1778.

5.3 Experimental for chapter 3

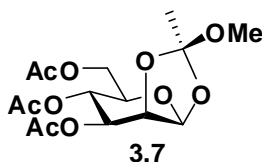
1,2,3,4,6-Penta-*O*-acetyl-D-mannopyranoside 3.6



D(+)-Mannose (15.0 g, 83.3 mmol) was dissolved in pyridine (300 mL), the reaction mixture cooled to 0 °C and acetic anhydride (190 mL) added slowly. The reaction mixture was stirred and allowed to warm to room temperature. After 20 h, t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (*R_f* 0.7) and complete consumption of starting material (*R_f* 0). The reaction mixture was poured onto ice-water (500 mL) and extracted with DCM (3 x 200 mL). The organic layers were combined and washed with hydrochloric acid (3 x 500 mL of a 1 M solution), sodium hydrogen carbonate (3 x 500 mL of a saturated solution), brine (2 x 500 mL), dried (MgSO₄), filtered and concentrated *in vacuo* to give 1,2,3,4,6-penta-*O*-acetyl-D- mannopyranoside

3.6 (32.0 g, 99 %) as a pale oil and mixture of anomers (7:1, α : β). δ_{H} (500 MHz, CDCl_3)²³ α anomer: 2.01, 2.05, 2.09, 2.17, 2.18 (15H, 5 x s, 5 x CH_3), 4.05-4.07 (1H, m, H-5), 4.08-4.11 (1H, m, H-6), 4.28 (1H, dd, $J_{5,6'}$ 4.8 Hz, $J_{6,6'}$ 12.4 Hz, H-6'), 4.33 (1H, m, H-2), 5.32-5.38 (2H, m, H-3, H-4), 6.08 (1H, s, H-1); β anomer: 2.01, 2.05, 2.09, 2.17, 2.18 (15H, 5 x s, 5 x CH_3), 3.81 (1H, m, H-5), 4.11-4.15 (1H, m, H-6), 4.29-4.33 (1H, m, H-6'), 5.13 (1H, dd, $J_{2,3}$ 3.2 Hz, $J_{3,4}$ 9.4 Hz, H-3), 5.32 (1H, m, H-4), 5.48-5.49 (1H, m, H-2), 5.86 (1H, br. s, H-1).

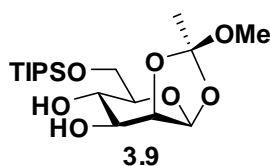
3,4,6-Tri-*O*-acetyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.7**



1,2,3,4,6-Penta-*O*-acetyl-D-mannopyranoside **3.6** (10.0 g, 24.4 mmol) was dissolved in DCM (20 mL) and the solution cooled to 0 °C. Hydrogen bromide (20 mL of a 33% in acetic acid solution) was added and the reaction mixture stirred at room temperature. After 2 h, t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.75) and complete consumption of starting material (R_f 0.7). Ice water (100 mL) was added and the reaction mixture extracted with DCM (2 x 100 mL). The organic extracts were combined and washed with sodium hydrogen carbonate (2 x 100 mL of a saturated solution), dried (MgSO_4), filtered and concentrated *in vacuo* to give 2,3,4,6-tetra-*O*-acetyl- α -D-mannopyranosyl bromide **5.2** (9.30 g, 93%) as a light yellow oil. Compound **5.2** (9.30 g, 22.6 mmol), methanol (1.83 mL, 45.2 mmol) and 2,4,6-collidine (3.9 mL, 29.4 mmol) were dissolved in DCM (60 mL) and the reaction mixture refluxed at 50 °C.

After 17 h, t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.6) and complete consumption of starting material (R_f 0.75). The reaction mixture was cooled to room temperature, water (100 mL) added and the reaction mixture extracted with DCM (100 mL). The combined organic layers were washed with sodium hydrogen carbonate (100 mL of a saturated solution) and brine (100 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was crystallised (diethyl ether) to give 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.7** (7.0 g, 85%) as white crystals, m.p. 114-115 °C [lit. 112-113°C],²⁴⁰ $[\alpha]_D^{20}$ -21 (c, 1.0 in $CHCl_3$) [lit. $[\alpha]_D^{25}$ -22.2 (c, 2.5 in $CHCl_3$)];⁴ δ_H (500 MHz, $CDCl_3$)²⁴¹ 1.60 (3H, s, CCH_3), 1.91, 1.92, 1.97 (9H, 3 x s, 3 x $OC(O)CH_3$), 3.14 (3H, s, $OC(CH_3)OCH_3$), 3.53 (1H, m, H-5), 4.00 (1H, m, H-6), 4.10 (1H, m, H-6'), 4.47 (1H, m, H-2), 5.01 (1H, m, H-3), 5.15 (1H, at, J 10 Hz, H-4), 5.35 (1H, s, H-1).

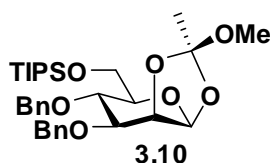
1,2-*O*-(1-methoxyethylidene)-6-*O*-triisopropylsilyl- β -D-mannopyranose **3.9**



3,4,6-Tri-*O*-acetyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.7** (2.00 g, 5.3 mmol) was dissolved in methanol (20 mL). Sodium (86.0 mg, 1.6 mmol) was added and the solution was stirred. After 35 min, t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.2) and complete consumption of starting material (R_f 0.6). The reaction mixture was concentrated *in vacuo*. The dried residue was taken up in dry THF (15 mL), stirred, imidazole (0.54 g, 8.0 mmol) was added. The mixture was

cooled to 0 °C and chloro-tri-*iso*-propylsilane (1.4 mL, 6.0 mmol) added. The reaction mixture was warmed to rt and stirred under an atmosphere of nitrogen. After 24 h t.l.c. (petrol:ethylacetate, 1:1) indicated formation of a major product (R_f 0.4) and consumption of starting material (R_f 0). The reaction mixture was diluted with ethyl acetate (20 mL), washed with ammonium chloride (2 x 15 mL of a saturated aqueous solution), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by flash column chromatography (ethyl acetate:petrol, 1:1) to afford 1,2-*O*-(1-methoxyethylidene)-6-*O*-triisopropylsilyl- β -D-mannopyranose **3.9** (1.0 g, 48%) as a yellow oil. $[\alpha]_D^{20} -15$ (c , 1.0 in CHCl_3); δ_H (500 MHz, CDCl_3)¹⁸⁵ 1.00 - 1.16 (m, 21 H), 1.60 (3H, s, CCH_3), 2.55 (d, J 6.85 Hz, 1 H), 3.14 (3H, s, $\text{OC}(\text{CH}_3)\text{OCH}_3$), 3.27 - 3.35 (m, 1 H), 3.53 - 3.65 (m, 3 H), 3.75 - 3.82 (m, 1 H), 3.88 (t, J =8.68 Hz, 2 H), 4.06 (dd, J =10.15, 4.28 Hz, 1 H) 4.49 (br. s., 1 H) 5.43 (s, 1 H).

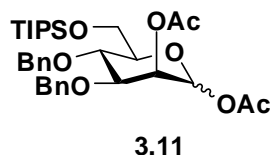
1,2-*O*-(1-methoxyethylidene)-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl- β -D-mannopyranose 3.10



1,2-*O*-(1-methoxyethylidene)-6-*O*-triisopropylsilyl- β -D-mannopyranose **3.9** (1.0 g, 2.5 mmol) was dissolved in DMF (10 mL) and sodium hydride (0.24 g, 10.0 mmol of a 50% dispersion in mineral oil) added in portions. The mixture was stirred under an atmosphere of nitrogen for 10 min and then benzyl bromide (1.2 mL, 10.0 mmol) added in portions. The reaction mixture was stirred for 18 h, after which time t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0.8) and consumption of starting material (R_f

0.4). Methanol (10 mL) was added and the solution stirred for 15 min then concentrated *in vacuo*. The residue was dissolved in ethylacetate (20 mL) and washed with water (2 x 15 mL). The aqueous washings were re-extracted with DCM (2 x 10 mL), the organics combined, dried (Na₂SO₄) and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 1:1) to afford 1,2-*O*-(1-methoxyethylidene)-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl-β-D-mannopyranose **3.10** (1.3 g, 92%) as a pale yellow oil. $[\alpha]_D^{20} + 53$ (*c*, 1.0 in CHCl₃)¹⁸⁵; δ_H (400 MHz, CDCl₃)¹⁸⁵ 0.9-1.09 (m, 21H), 1.71 (s, 3H, CH₃), 3.14 (s, 3H OCH₃), 3.87-3.97 (m, 3H), 4.01 (m, 1H, 5-H), 4.48 (dd, *J* = 2.8 Hz, 4.0 Hz, 1H, 2-H), 5.42 (d, *J* = 2.4 Hz, 1H, 1-H).

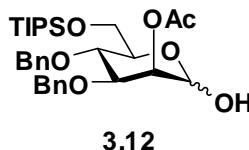
1,2-di-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl-α/β-D-mannopyranose 3.11



To a mixture of acetic acid (94mL), water (48 mL), and acetone (70 mL) 1,2-*O*-(1-methoxyethylidene)-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl-β-D-mannopyranose **3.10** (4.2 g, 7.3 mmol) was added and stirred for 18 hr at room temperature. The clear solution was concentrated *in vacuo* and co-evaporated with toluene (50 mL) two times. The residue was dissolved in anhydrous pyridine/acetic anhydride (1:1, 25 mL) and stirred for 7 h at room temperature. Concentrated *in vacuo* and purified by flash column chromatography with (petrol:ethyl acetate, 4:1) gave 1,2-di-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl-α/β-D-mannopyranose **3.11** (3.0 g, 68%) as an anomeric mixture. *R_f* 0.7 (petrol:ethyl acetate, 4:1). δ_H (400 MHz, CDCl₃)¹⁸⁵ 1.08-1.09 (m, 21H), 2.05 (s, 3H,

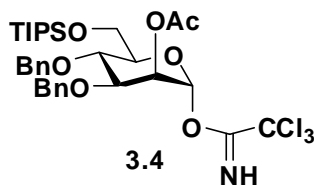
COCH₃), 2.13 (s, 3H, COCH₃), 3.68-3.71 (m, 1H, 5-H), 3.91-4.13 (m, 4H), 4.57-4.59 (m, 1H), 4.68-4.76 (m, 2H), 4.90-4.93 (m, 1H), 5.32 (dd, 1H, *J* = 2.4 Hz, 3.2 Hz, H-2), 6.07 (d, 1H, *J* = 2.0 Hz, H-1), 7.31-7.32 (m, 10H, Ar).

2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl- α/β -D-mannopyranose **3.12**



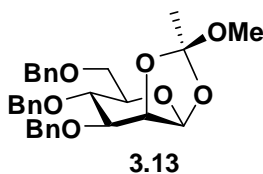
A solution of 1,2-di-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl- α/β -D-mannopyranose **3.11** (3.0g, 5.0 mmol) and hydrazinium acetate (0.60 g, 5.9 mmol) in dry N,N-dimethylformamide (60 mL) was heated for 4 h at 50 °C under nitrogen atmosphere. After cooling to room temp, water (500 mL) was added and the mixture was extracted with ethyl acetate (3 × 150 mL). The combined organic extracts were washed with water (2 × 150 mL), dried with Na₂SO₄, and concentrated *in vacuo*. Purification of the residue by flash chromatography (hexane/ethyl acetate, 4:1) gave 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl- α/β -D-mannopyranose **3.12** (2.6 g, 95%, colorless syrup) as an anomeric mixture. *R*_f = 0.69 (petrol:ethyl acetate, 4:1). δ_{H} (400 MHz, CDCl₃)¹⁸⁵ 1.08-1.07 (m, 21H), 2.12 (s, 3H, COCH₃), 3.88- 4.05 (m, 5H), 4.56-4.59 (m, 1H), 4.65-4.73 (m, 2H), 4.90-4.92 (m, 1H), 5.18 (s., 1H), 5.34 (d, 1H, *J* = 2.0 Hz, 1-H), 7.32-7.31 (m, 10H, Ar).

2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl- α -D-mannopyranose trichloroacetimidate **3.4**



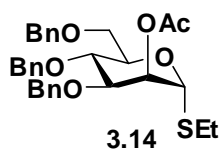
2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-isopropylsilyl- α/β -D-mannopyranose **3.12** (0.17 g, 0.3 mmol) was dissolved in DCM (1.5 mL). 1,1,1-Trichloroacetonitrile (1.6 mL) was added and the reaction mixture cooled to 0 °C. DBU (14 μ l, 0.09 μ mol) was added and the solution was stirred and allowed to warm to room temperature under nitrogen atmosphere. After 1h, t.l.c (petrol:ethyl acetate, 4:1) indicated formation of a major product (R_f 0.5) and complete consumption of starting material (R_f 0.3). The reaction mixture was filtered through Celite[®] and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 4:1) to give 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl- α -D-mannopyranose trichloroacetimidate **3.4** (0.18 g, 85%) as a colorless foam, $[\alpha]_D^{20} + 48$ (c , 1.0 in CHCl_3); [lit. $[\alpha]_D^{22} + 43.4$ (c , 2.2 in CH_2Cl_2)¹⁸⁵; δ_H (400 MHz, CDCl_3)³ 1.08-1.14 (m, 21H), 2.15 (s, 3H, COCH_3), 3.85 (dd, J 1.5, 9.8 Hz, 1H), 3.95 (d, J 11.3 Hz, 1H), 4.09-4.05 (m, 2 H), 4.17 (t, J 9.8 Hz, 1H), 4.62-4.57 (m, 1H), 4.71 (d, J 10.7 Hz, 1H), 4.75 (d, J 11.3 Hz, 1H), 4.93 (d, J 10.4 Hz, 1H), 5.46 (dd, J 2.1, 3.1 Hz, 1H), 6.26 (d, J 1.8 Hz, 1H), 7.32-7.33 (m, 10 H, Ar-H), 8.63 (s, 1H, NH).

3,4,6-Tri-*O*-benzyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.13**



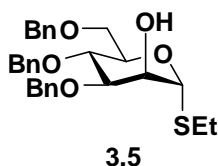
3,4,6-Tri-*O*-acetyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.7** (6.00 g, 16.9 mmol) was dissolved in methanol (60 mL). Sodium methoxide (27.0 mg, 5.0 mmol) was added and the solution stirred. After 35 min, t.l.c. (petrol:ethyl acetate, 1:1) indicated formation of a major product (R_f 0) and complete consumption of starting material (R_f 0.6). The reaction mixture was concentrated *in vacuo*. The dried residue was taken up in dry DMF (60 mL), stirred and the solution cooled to 0 °C. Benzyl bromide (8.90 mL, 75.0 mmol) was added followed by slow addition of sodium hydride (4.70 g of a 60% mineral oil dispersion, 99.4 mmol). After 17 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated formation of a major product (R_f 0.45) and complete consumption of starting material (R_f 0). Methanol (20 mL) was added slowly until bubbling ceased. The reaction mixture was extracted with DCM (2 x 200 mL), washed with water (2 x 100 mL), brine (100 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 3:1) to yield 3,4,6-tri-*O*-benzyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.13** as a white solid (7.10 g, 88%), m.p. 73-74 °C [lit. 71-72 °C]; $[\alpha]_D^{20} +31$ (c, 1.0 in CHCl₃) [lit. $[\alpha]_D^{20} +34$ (c, 1.35 in CHCl₃)]; δ_H (500 MHz, CDCl₃) ^{184,241-243} 1.76 (3H, s, CCH₃), 3.31 (3H, s, OCH₃), 3.41-3.47 (1H, m, H-5), 3.70-3.78 (3H, m, H-3, H-6, H-6'), 3.93 (1H, at, H-4), 4.40 (1H, m, H-2), 4.55, 4.61 (2H, m, PhCH₂), 4.61, 4.90 (2H, m, PhCH₂), 4.80 (2H, m, PhCH₂), 5.36 (1H, d, H-1), 7.24-7.41 (15H, m, 15 x Ar-H).

Ethyl -2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside **3.14**



3,4,6-tri-*O*-benzyl-1,2-*O*-(1-exo-methoxyethylidene)- β -D-mannopyranoside **3.13** (6.00 g, 12.2 mmol) was dissolved in dry MeCN (40 mL). The mixture was stirred for 60 min before ethanethiol (2.91 mL, 40.4 mmol) and HgBr₂ (220 mg, 60 mmol) were added. The suspension was stirred at 60 °C. After 24 h, t.l.c. (petrol:ethyl acetate, 3:1) indicated formation of a major product (*R_f* 0.75) and complete consumption of starting material (*R_f* 6). Diluted with DCM (50 mL), washed with 5% NaOH (50 mL), dried (MgSO₄) and concentrated. The residue was purified by column chromatography (petrol:ethyl acetate, 3:1) to give Ethyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside **3.14** as a colourless oil (5.10 g, 78%). δ_{H} (500 MHz, CDCl₃)¹⁸⁴ 1.28 (3H, t, *J* 10 Hz, CH₂CH₃), 2.17 (3H, s, OC(O)CH₃), 2.55 - 2.70 (2H, m, CH₂CH₃), 3.68 (1H, m, H-6), 3.84 (1H, m, H-6'), 3.90 - 3.98 (2H, m, H-3, H-4), 4.14 - 4.20 (1H, m, H-5), 4.45 - 4.56 (3H, m, CH₂Ph), 4.68-4.72 (2H, m, CH₂Ph), 4.85 (1H, d, *J* 10 Hz, CH₂Ph), 5.32 (1H, s, H-1), 5.44 (1H, d, *J* 1.5 Hz, H-2), 7.12 - 7.38 (15H, m, Ar-H); ¹³C NMR (125 MHz, CDCl₃): 14.9 (CH₂CH₃), 21.2 (OC(O)CH₃), 25.5 (CH₂CH₃), 68.9 (C-6), 70.6, 71.8, 74.6 (C-3, C-4, C-5), 71.9, 73.4, 75.2 (CH₂Ph), 78.6 (C-2), 82.5 (C-1), 127.6, 127.7, 127.8, 127.9, 128.2, 128.3, 128.5, 137.7, 138.2, 138.4, (Ar-C), 170.5 (C=O).

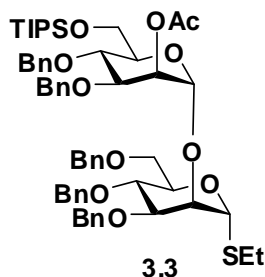
Ethyl 3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside 3.5



Ethyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside **3.14** (3.2 g, 1.90 mmol) was dissolved in MeOH (30 mL) and NaOMe (0.03g, 0.6 mmol) was added. After

1 h, t.l.c. (petrol:ethyl acetate, 3:1) indicated formation of a major product (R_f 0.50) and complete consumption of starting material (R_f 0.75). The reaction mixture was filtered, concentrated and purified by column chromatography (petrol:ethyl acetate, 3:1) to give Ethyl 3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside **3.5** as a colourless oil (2.95 g, 100%). δ_H (500 MHz, $CDCl_3$)¹⁸⁴ 1.28 (3H, t, J .10 Hz, CH_2CH_3), 2.53 - 2.71 (3H, m, CH_2CH_3 , OH), 3.67 (1H, dd, J .1.9, 10 Hz, H-6), 3.79 (1H, m, H-6'), 3.84 (1H, m, H-3), 3.90 (1H, t, J . 10 Hz, H-4), 4.09-4.12 (1H, m, H-2), 4.16-4.20 (1H, m, H-5), 4.50 (2H, d, J .10Hz CH_2Ph), 4.65-4.72 (3H, m, CH_2Ph), 4.82 (1H, d, J . 10.9 Hz, CH_2Ph), 5.39 (1H, s, H-1), 7.16 - 7.39 (15H, m, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): 14.8 (CH_2CH_3), 24.9 (CH_2CH_3), 68.9 (C- 6), 69.9, 71.5, 74.6 (C-3, C-4, C-5), 72.1, 73.4, 75.1 (CH_2Ph), 80.5 (C-2), 83.4 (C-1), 127.6, 127.7, 127.8, 127.9, 128.0, 128.3, 128.4, 128.6, 137.7, 138.3, 138.4 (Ar-C).

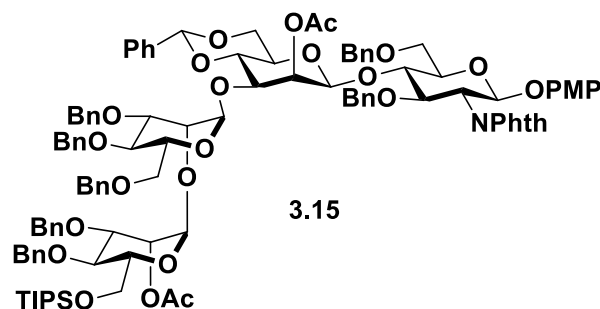
Ethyl 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl-(1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl-thio- α -D-mannopyranoside **3.3**



Ethyl 3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside **3.5** (1.0 g, 2.0 mmol) and 2-*O*-acetyl-3,4-di-*O*-benzyl-1-*O*-trichloroacetimidyl-6-*O*-triisopropylsilyl- α/β -D-mannopyranosyl **3.4** (2.1 g, 3.0 mmol) were dissolved in dry DCM (20 mL) and transferred to a flame-dried round-bottomed flask containing activated 4Å molecular

sieves (2.00 g). The solution was cooled to -10 °C and stirred under an atmosphere of nitrogen. TMSOTf (13.0 µl, 0.08 mmol) was added and the temperature was allowed to rise to 0 °C. After 25 min, t.l.c. (petrol:ethyl acetate, 9:1) indicated formation of a major product (R_f 0.7) and complete consumption of Ethyl 3,4,6-tri-*O*-benzyl-1-thio- α -D-mannopyranoside **3.5**. (R_f 0.2). Triethylamine was added and the solution was stirred for a further 10 min. The reaction mixture was filtered through Celite[®], washed with sodium hydrogen carbonate (10 mL of a saturated solution), dried (Na₂SO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol: ethyl acetate, 20:1) to afford compound **3.3** (1.6 g, 61%) as yellow oil. $[\alpha]_D^{20}$ +8.3 (*c*, 1.0 in CHCl₃); [lit. $[\alpha]_D^{20}$ + 6.43 (*c*, 0.42 in CH₂Cl₂)⁴⁹; ¹H NMR δ_H (400 MHz, CDCl₃)⁵ 0.98 - 1.18 (21H, m), 1.25 (t, *J* 7.43 Hz, 3 H), 2.08 (s, 3 H), 2.58 (m, 2 H), 3.64 - 3.71 (m, 1 H), 3.71 - 3.81 (m, 2 H), 3.81 - 4.07 (m, 7 H), 4.11 (br. s., 2 H), 4.39 - 4.58 (m, 3 H), 4.59 - 4.69 (m, 5 H), 4.86 (m, 2H), 5.10 (s, 1 H), 5.30 (s, 1 H), 5.47 (br. s., 1 H), 7.14 - 7.39 (m, 25 H).

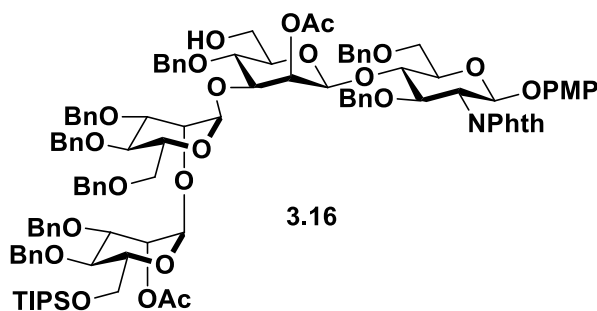
***p*-Methoxyphenyl 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl- (1→2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1→3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside 3.15**



A solution of *p*-methoxyphenyl 2-*O*-acetyl-4,6-*O*-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6 di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.21** (0.07 g, 0.08 mmol), **3.3** (0.10 g, 0.1 mmol) and tri-*tert*-butylpyrimidine (0.11 g, 0.45 mmol) in DCM (2 mL) was added to a flame-dried round bottom flask containing 3Å molecular sieves (0.2 g). The solution was cooled to 0 °C under an atmosphere of nitrogen, stirred for 30 min and methyl trifluoromethanesulfonate (0.05 mL, 0.45 mmol) was added. After 18 h t.l.c. (petrol: ethyl acetate, 3:2) indicated formation of a major product (*R_f* 0.4) and complete consumption of alcohol starting material **2.21** (*R_f* 0.20). Triethylamine (0.10 mL) was added and the reaction mixture stirred for 10 min before being filtered through Celite® and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol: ethyl acetate, 3:2) to afford compound *p*-Methoxyphenyl 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl-(1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.15** (80 mg, 55%) as a yellow foam. $[\alpha]_D^{20}$ -2.1 (*c*, 1.0 in CHCl₃); δ_H (400 MHz, CDCl₃) 0.94 - 1.06 (m, 21 H), 2.07 (s, 3 H), 2.04 (s, 3 H), 3.04 (m, 2 H), 3.45 - 3.54 (m, 3 H), 3.54 - 3.64 (m, 3 H), 3.65 - 3.74 (m, 7 H), 3.74 - 3.92 (m, 6 H), 3.92 - 4.03 (m, 4 H), 4.03 - 4.17 (m, 4 H), 4.26 - 4.53 (m, 8 H), 4.53 - 4.74 (m, 10 H), 4.78 - 4.91 (m, 4 H), 5.16 (m, 3 H), 5.43 (d,

$J=5.87$ Hz, 3 H), 5.57 (d, $J=8.61$ Hz, 1 H), 6.70 (d, $J=9.00$ Hz, 2 H), 6.80 (m, 3 H), 6.88 - 6.97 (m, 3 H), 7.02 (m, 3 H), 7.14 - 7.41 (m, 35 H), 7.68 (br. s., 4 H); ^{13}C NMR (125 MHz, CDCl_3) 12.1, 18.0, 18.1, 20.9, 21.0, 29.7, 55.6, 62.0, 66.6, 68.1, 68.4, 68.7, 69.2, 69.4, 71.0, 71.8, 72.0, 72.2, 72.6, 73.2, 73.3, 73.6, 74.4, 74.5, 74.7, 75.2, 76.7, 77.0, 77.2, 77.3, 78.0, 78.5, 78.5, 97.6, 98.0, 99.2, 99.7, 101.3, 114.4, 118.6, 123.3, 125.7, 127.2, 127.4, 127.5, 127.6, 127.7, 127.8, 127.9, 127.96, 127.98, 128.0, 128.1, 128.2, 128.2, 128.3, 128.3, 128.4, 128.5, 128.9, 131.5, 133.8, 137.1, 137.9, 138.3, 138.3, 138.5, 138.5, 138.5, 139.0, 150.8, 155.4, 169.6, 170.0.; HRMS (ES^+) Calculated For $\text{C}_{108}\text{H}_{121}\text{NSiNaO}_{25}$ (MNa^+) 1883.7928. Found (MNa^+):1883.7990.

***p*-Methoxyphenyl 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside 3.16**

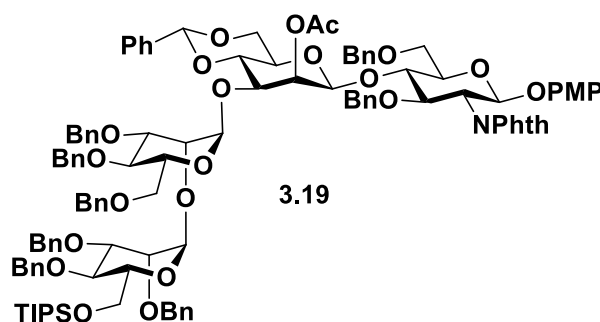


A solution of *p*-Methoxyphenyl 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-

β -D-glucopyranoside **3.15** (0.06 g, 0.03 mmol) in DCM (2.5 mL) was added to a flame-dried round bottom flask containing 3Å molecular sieves (0.12 g). The solution was stirred for 30 min under an atmosphere of nitrogen then cooled to -78 °C. Triethylsilane (14 μ L, 0.09 mmol) and dichlorophenyl borane (14 μ L, 0.11 mmol) were added and stirred the reaction mixture at -78 °C. After 30 min t.l.c. (petrol:ethyl acetate, 7:3) showed formation of a single product (Rf 0.3) and consumption of starting material (Rf 0.5). Triethylamine (0.1 mL) and methanol (0.1 mL) were added, the reaction mixture diluted with DCM (4 mL), washed with sodium hydrogen carbonate (2 x 5 mL of a saturated solution), dried (Na₂SO₄), filtered and concentrated *in vacuo*. The residue was evaporated with methanol five times at 50 °C before being purified by flash column chromatography (7:3 petrol: ethyl acetate) to obtain *p*-Methoxyphenyl 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.16** (0.05 g, 83%) as a yellow foam. $[\alpha]_D^{20} +1.5$ (*c*, 1.0 in CHCl₃); δ_H (400 MHz, CDCl₃) 0.91 - 1.05 (m, 21 H), 2.01 - 2.14 (m, 6 H), 2.98 - 3.08 (m, 1 H), 3.31 (m, 1 H), 3.51 - 3.64 (m, 4 H), 3.64 - 3.82 (m, 11 H), 3.82 - 3.96 (m, 5 H), 3.99 (dd, *J*=9.59, 2.54 Hz, 2 H), 4.09 (m, 3 H), 4.20 - 4.32 (m, 2 H), 4.32 - 4.53 (m, 7 H), 4.56 - 4.71 (m, 8 H), 4.76 - 4.92 (m, 4 H), 5.03 (s, 2 H), 5.12 (s, 1 H), 5.36 - 5.48 (m, 3 H), 5.58 (d, *J*=8.61 Hz, 2 H), 6.69 (d, *J*=9.00 Hz, 2 H), 6.79 (d, *J*=8.61 Hz, 3 H), 6.94 (m, 3 H), 7.01 (m, 3 H), 7.17 - 7.39 (m, 32 H), 7.67 (br. s., 4 H); δ_C (125 MHz, CDCl₃) 12.0, 17.9, 18.0, 21.0, 21.0, 24.6, 55.6, 61.6, 62.1, 67.9, 68.8, 69.3, 71.3, 71.8, 72.0, 72.8, 73.2, 73.4, 73.5, 74.1, 74.4, 74.5, 74.6, 74.8, 75.2, 75.4, 76.7, 77.0, 77.3, 77.8, 77.9, 78.7, 97.6, 98.2, 100.2, 101.2, 114.4, 115.3, 118.6, 123.4, 127.4,

127.5, 127.5, 127.8, 127.9, 128.0, 128.1, 128.2, 128.2, 128.2, 128.3, 128.5, 133.6, 133.8, 137.8, 137.8, 138.2, 138.3, 138.5, 138.6, 138.9, 150.8, 169.6, 170.1.

p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl-(1 \rightarrow 2)-3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.19*

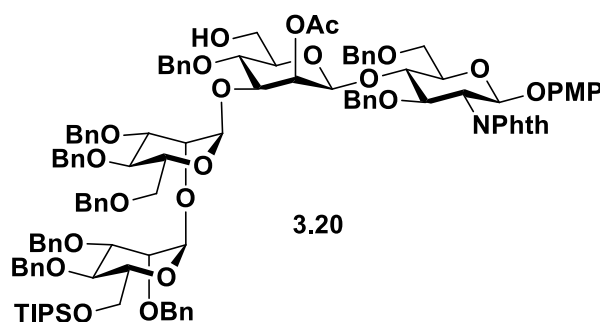


A solution of *p*-methoxyphenyl 2-*O*-acetyl-4,6-*O*-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6 di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **2.21** (0.22 g, 0.25 mmol), and **3.18** (0.32 g, 0.29 mmol) in DCM (10 mL) was added to a flame-dried round bottom flask containing 3Å molecular sieves (0.5 g). The solution was cooled to -20 °C under an atmosphere of nitrogen, stirred for 30 min and NIS (0.07 g, 0.30 mmol) was added. After 10 min, triflic acid (2.6 μ L, 0.03 mmol) was added. After a further 30 min t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of a major product (R_f 0.5) and the complete consumption of alcohol starting material **2.21** (R_f 0.3). Triethylamine (0.1 mL) was added and the reaction mixture stirred for 10 min before being filtered through Celite[®]. The filtrate was diluted with DCM (10 mL) and washed

with sat. aqueous NaHCO_3 (10 mL), brine, dried over anhydrous Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol: ethyl acetate, 2:1) to afford *p*-Methoxyphenyl 2,3,4-tri-O-benzyl-6-O-tri-iso-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6-tri-O-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-O-acetyl-4,6-O-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.19** (0.31 g, 65%) as a white foam. $[\alpha]_{\text{D}}^{20} +2$ (c, 1.0 in CHCl_3); ν_{max} (KBr disk) 1753, 1712 (s, C=O) cm^{-1} ; δ_{H} (400 MHz, CDCl_3) 1.02-1.26 (21H, m, 3 x $\text{CH}(\text{CH}_3)_2$), 2.09 (3H, s, CH_3), 3.03-3.08 (1H, m, H-5b), 3.43-3.46 (1H, b, H-6b), 3.65-3.71 (5H, m, H-3b, H-5a, H-6c, H-6'c), 3.72 (3H, s, OCH_3), 3.72-3.89 (13H, m, H-4b, H-6a, H-6'a, H-3c, H-4c, H-5c, H-3d, H-4d, H-5d, H-6d, H-6'd), 3.97- 4.17 (5H, m, H-4a, H-6'b, H-2c, H-2d, H-3d), 4.28-4.34 (1H, m, H-3a), 4.40-4.68 (15H, m, H-2a, 14 x PhCH_2), 4.71 (1H, s, H-1c), 4.80-4.92 (4H, m, 4 x PhCH_2), 5.17 (1H, s, H-1b), 5.40 (1H, s, H-1d), 4.42-4.45 (1H, m, H-2b), 5.50 (1H, s, $\text{PhCH}(\text{O})$), 5.61 (1H, d, $J_{1,2}$ 8.2 Hz, H-1a), 6.69-6.72 (2H, m, 2 x Ar-H), 6.79-6.83 (2H, m, 2 x Ar-H), 6.88-6.95 (3H, m, 3 x Ar-H), 7.02-7.04 (2H, m, 2 x Ar-H), 7.33-7.40 (40H, m, 40 x Ar-H), 7.68-7.80 (4H, m, 4 x Ar-H); δ_{C} (100 MHz, CDCl_3) 12.0 (d, $\text{CH}(\text{CH}_3)_2$), 18.0 (q, $\text{CH}(\text{CH}_3)_2$), 21.1 (s, CH_3), 55.6 (q, OCH_3), 55.7 (d, C-2a), 62.5 (t, C-6c), 66.3 (d, C-5a), 66.5 (d, C-5b), 68.1 (t, C-6a), 68.4 (t, C-6b), 69.5 (t, C-6d), 71.0 (d, C-2b), 71.4, 71.5, 72.0, 72.3, 73.0, 73.7, 74.4 (8 x t, 8 x PhCH_2), 72.8 (d, C-5d), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.4 (d, C-4d), 74.5 (d, C-2c), 74.7 (d, C-4c), 75.1 (d, C-2d), 76.9 (d, C-3a), 78.5 (d, C-4a), 79.2 (d, C-4b), 79.6 (d, C-2c), 80.2 (d, C-3d), 97.4 (d, C-1a), 97.7 (d, C-1d), 99.2 (d, C-1c), 99.7 (d, C-1b), 101.3 (d, $\text{PhCH}(\text{O})$), 114.3, 118.6, 123.4, 127.2, 125.63, 127.2, 127.3, 127.4, 127.4, 127.5, 127.5, 127.6, 127.7, 127.7, 127.8, 127.9, 128.0, 128.1, 128.1, 128.2, 128.3,

128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.4, 129.6, 131.6, 133.8 (32 x d, 54 x Ar-C), 136.9, 137.1, 137.9, 138.0, 138.1, 138.4, 138.5, 138.6, 138.6, 138.8, 139.1 (11 x s, 12 x Ar-C), 151.0, 155.5, 169.6 (3 x s, 3 x C=O); HRMS (ES⁺) Calculated For C₁₁₃H₁₂₅NO₂₄Si (MH⁺) 1908.84. Found (MH⁺) 1908.84.

p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.20*



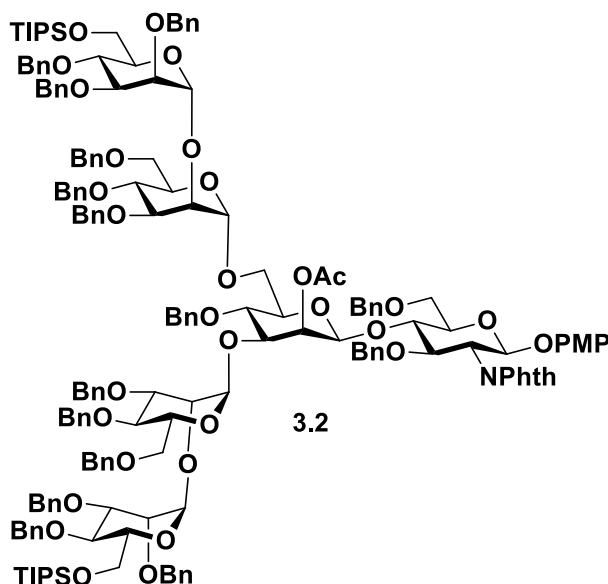
A solution of *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl- (1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4,6-*O*-benzylidene- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.19** (0.37 g, 0.20 mmol) in DCM (5 mL) was added to a flame-dried round bottom flask containing 3Å molecular sieves (1.0 g). The solution was stirred for 30 min under an atmosphere of nitrogen, and then cooled to -78 °C. Triethylsilane (0.14 mL, 0.86 mmol) and dichlorophenyl borane (90 μ L, 0.67 mmol) were added and the reaction mixture was stirred at -78 °C. After 30 min, t.l.c. (petrol: ethyl acetate, 2:1)

showed the formation of a single product (Rf 0.3) and the consumption of starting material (Rf 0.5). Triethylamine (5 mL) and methanol (5 mL) were added, and the reaction mixture diluted with DCM (20 mL), washed with aqueous sodium hydrogen carbonate (2 x 10 mL of a saturated solution), dried (Na₂SO₄), filtered, and concentrated *in vacuo*. The residue was evaporated with methanol five times at 50 °C before being purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 2)-3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside

3.20 (0.29 g, 78%) as a white foam. $[\alpha]_D^{20} +3.5$ (*c*, 1.0 in CHCl₃); ν_{\max} (KBr disk) 1747, 1715, 1634 (s, C=O) cm⁻¹; δ_H (400 MHz, CDCl₃) 1.02-1.26 (21H, m, 3 x CH(CH₃)₂), 2.09 (3H, s, CH₃), 3.03-3.08 (1H, m, H-5b), 3.43-3.46 (1H, b, H-6b), 3.65-3.71 (5H, m, H-3b, H-5a, H-6c, H-6'c), 3.72 (3H, s, OCH₃), 3.72-3.89 (13H, m, H-4b, H-6a, H-6'a, H-3c, H-4c, H-5c, H-3d, H-4d, H-5d, H-6d, H-6'd), 3.97- 4.17 (5H, m, H-4a, H-6'b, H-2c, H-2d, H-3d), 4.28-4.34 (1H, m, H-3a), 4.40-4.68 (15H, m, H-2a, 14 x PhCH₂), 4.71 (1H, s, H-1c), 4.80-4.92 (4H, m, 4 x PhCH₂), 5.17 (1H, s, H-1b), 5.40 (1H, s, H-1d), 5.42-5.45 (1H, m, H-2b), 5.50 (1H, s, PhCH(O)), 5.61 (1H, d, *J*_{1,2} 8.2 Hz, H-1a), 6.69-6.72 (2H, m, 2 x Ar-H), 6.79-6.83 (2H, m, 2 x Ar-H), 6.88-6.95 (3H, m, 3 x Ar-H), 7.02-7.04 (2H, m, 2 x Ar-H), 7.33-7.40 (40H, m, 40 x Ar-H), 7.68-7.80 (4H, m, 4 x Ar-H); δ_C (100 MHz, CDCl₃) 12.0 (d, CH(CH₃)₂), 18.0 (q, CH(CH₃)₂), 21.1 (s, CH₃), 55.6 (q, OCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 66.3 (d, C-5a), 66.5 (d, C-5b), 68.1 (t, C-6a), 68.4 (t, C-6b), 69.5 (t, C-6d), 71.0 (d, C-2b), 71.4, 71.5, 71.7, 72.0, 72.3, 73.0, 73.7, 74.1, 74.4 (9 x t, 9 x PhCH₂), 72.8 (d, C-5d), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.4 (d, C-4d), 74.5 (d, C-2c),

74.7 (d, C-4c), 75.1 (d, C-2d), 76.9 (d, C-3a), 78.5 (d, C-4a), 79.2 (d, C-4b), 79.6 (d, C-2c), 80.2 (d, C-3d), 97.6 (d, C-1a), 98.2 (d, C-1d), 98.5 (d, C-1c), 101.4 (d, C-1b), 114.3, 118.6, 123.2, 123.3, 123.4, 127.2, 127.3, 127.4, 127.4, 127.5, 127.5, 127.6, 127.7, 127.7, 127.8, 127.9, 128.0, 128.1, 128.1, 128.2, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.4, 129.6, 131.6, 133.8 (32 x d, 54 x Ar-C), 136.9, 137.1, 137.9, 138.0, 138.1, 138.4, 138.5, 138.6, 138.6, 138.8, 139.1 (11 x s, 12 x Ar-C), 151.0, 155.5, 169.6 (3 x s, 3 x C=O); HRMS (ES⁺) Calculated For C₁₁₃H₁₂₈NO₂₄Si (MH⁺) 1911.8629. Found (MH⁺) 1911.8603.

***p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl-(1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside 3.2**



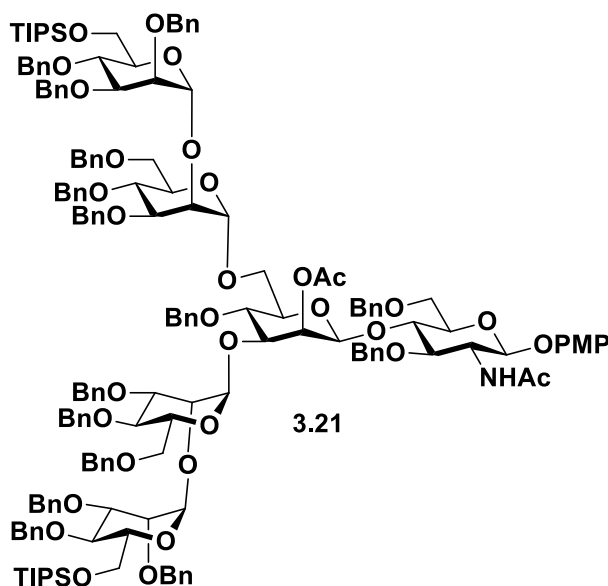
A solution of *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl-(1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)-2-*O*-acetyl-4-*O*-benzyl- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.20** (0.15 g, 0.08 mmol), and **3.18** (0.17 g, 0.16 mmol) in DCM (10 mL) was added to a flame-dried round bottom flask containing 3Å molecular sieves (0.3 g). The solution was cooled to -20 °C under an atmosphere of nitrogen, stirred for 30 min and NIS (0.04 g, 0.19 mmol) was added. After 10 min, triflic acid (1.0 μ L, 0.01mmol) was added. After a further 30 min t.l.c. (petrol: ethyl acetate, 2:1) indicated the formation of a major product (Rf 0.6) and the complete consumption of alcohol starting material **3.20** (Rf 0.3). Triethylamine (0.1 mL) was added and the reaction mixture stirred for 10 min before being filtered through Celite[®]. The filtrate was diluted with DCM (10 mL) and washed with sat.aqueous NaHCO₃ (10 mL), brine, dried over anhydrous Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol:ethyl acetate, 2:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-

6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.2** (0.24 g, 72%) as a white foam. $[\alpha]_D^{20} +11$ (c, 1.0 in CHCl₃); ν_{\max} (KBr disk) 1716, 1635 (s, C=O) cm⁻¹; δ_H (400 MHz, CDCl₃) 1.02-1.10 (42H, m, 6 x CH(CH₃)₂), 2.09 (3H, s, CH₃), 3.24-3.29 (1H, m, H-5b), 3.40-3.43 (1H, m, H-6b), 3.49-4.19 (31H, m, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H-4c', H-5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd'), 3.72 (3H, s, OCH₃), 4.23-4.29 (1H, m, H-3a), 4.30-4.57 (20H, m, H-2a, 19 x PhCH₂), 4.61- 4.69 (5H, m, 5 x PhCH₂) 4.71-4.82 (6H, m, H-1c, H-1c', 4 x PhCH₂), 4.86-4.92 (2H, m, 2 x PhCH₂), 5.03(1H, s, H-1b), 5.10 (1H, s, H-1d'), 5.21 (1H, s, H-1d), 5.42-5.45(1H, m, H-2b), 5.50 (1H, d, $J_{1,2}$ 8.2 Hz, H-1a), 6.69-6.72 (2H, m, 2 x Ar-H), 6.79-6.83 (2H, m, 2 x Ar-H), 6.88-6.95 (3H, m, 3 x Ar-H), 7.02-7.04 (2H, m, 2 x Ar-H), 7.33-7.40 (70H, m, 70 x Ar-H), 7.68-7.80 (4H, m, 4 x Ar-H); δ_C (100 MHz, CDCl₃) 12.0 (d, CH(CH₃)₂), 18.0 (q, CH(CH₃)₂), 21.1 (s, CH₃), 55.6 (q, OCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 62.9 (t, C-6c'), 67.1 (d, C-5a), 68.1 (d, C-5b), 69.1 (t, C-6a), 69.2 (t, C-6b), 69.3 (t, C-6d), 69.4 (t, C-6d'), 71.0 (d, C-2b), 71.4, 71.5, 71.8, 71.9, 72.0, 72.1, 72.2, 72.3, 72.3, 73.0, 73.7, 74.3, 74.5, 74.6, 74.7 (15 x t, 15 x PhCH₂), 72.8 (d, C-5d), 73.1 (d, C-5d'), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.3 (d, C-5c'), 74.4 (d, C-4d), 74.5 (d, C-4d'), 74.5 (d, C-2c), 74.6 (d, C-2c'), 74.7 (d, C-4c), 74.8 (d, C-4c'), 75.1 (d, C-2d), 76.3 (d, C-2d'), 76.9 (d, C-3a), 78.8 (d, C-4a), 79.6 (d, C-4b), 79.7 (d, C-2c), 80.0 (d, C-2c'), 80.1 (d, C-3d), 80.2 (d, C-3d'), 97.4 (d, C-1a), 98.2 (d, C-1d),

98.6 (d, C-1d'), 99.0 (d, C-1c), 99.6 (d, C-1c'), 101.6 (d, C-1b), 114.3, 118.6, 126.7, 126.8, 126.9, 127.0, 127.1, 127.1, 127.2, 127.2, 127.3, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.6, 127.6, 127.7, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.2, 128.2, 128.2, 128.2, 128.3, 128.3, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.4, 129.6, 131.6, 133.8 (50 x d, 84 x Ar-C), 136.9, 137.1, 137.9, 138.0, 138.0, 138.1, 138.2, 138.2, 138.3, 138.4, 138.5, 138.6, 138.6, 138.7, 138.8, 139.0, 139.1 (17 x s, 18 x Ar-C), 151.0, 155.2, 169.6 (3 x s, 3 x C=O).

The stereochemistry of the anomeric linkages was confirmed by obtaining the H^1-C^{13} coupling constants from C-coupled HSQC experiments. They were as follows: C(1a)-H(1a) 168 Hz; C(1b)-H(1b) 162 Hz; C(1c)-H(1c) 173 Hz; C(1d)-H(1d) 175 Hz; C(1c')-H(1c') 172 Hz; C(1d')-H(1d') 174 Hz.

***p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl- [2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido- 3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside 3.21**



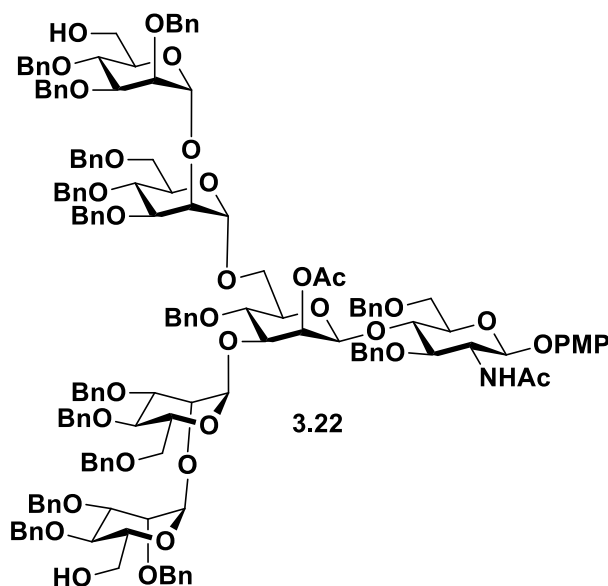
p-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside **3.2** (0.08 g, 0.03 mmol) was dissolved in a mixture of methanol (4 mL) and ethylene diamine (2 mL), and the solution was refluxed under an atmosphere of nitrogen. After 16 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of a single product (*R_f* 0.25) and the complete consumption of starting material (*R_f* 0.60). The reaction mixture was concentrated *in vacuo* and co-distilled five times with toluene (5 mL). The residue was dissolved in pyridine (2 mL), cooled to 0°C and acetic anhydride (0.7 mL) was added. The reaction mixture was then stirred at rt under an atmosphere of nitrogen. After 20 h, t.l.c. (petrol:ethyl acetate, 2:1) indicated the formation of a single product (*R_f* 0.35) and the complete consumption of amine intermediate (*R_f* 0.25). The reaction mixture was concentrated *in vacuo*, the residue

dissolved in DCM (10 mL), washed with water (10 mL), sodium hydrogen carbonate (2 x 10 mL of a saturated aqueous solution), dried (Na₂SO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol: ethyl acetate, 2:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-mannopyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside **3.21** (0.06 g, 71%) as a pale yellow foam. $[\alpha]_D^{20} +9$ (c, 1.0 in CHCl₃); ν_{\max} (KBr disk) 1716, 1635 (s, C=O) cm⁻¹; δ_H (400 MHz, CDCl₃) 1.01-1.10 (42H, m, 6 \times CH(CH₃)₂), 1.45 (3H, s, COCH₃), 1.85 (3H, s, CH₃), 3.21-3.27 (1H, m, H-5b), 3.32-3.43 (1H, m, H-6b), 3.49-4.19 (31H, m, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H-4c', H-5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd'), 3.72 (3H, s, OCH₃), 4.23-4.29 (1H, m, H-3a), 4.30-4.57 (20H, m, H-2a, 19 \times PhCH₂), 4.61- 4.69 (5H, m, 5 \times PhCH₂) 4.71-4.82 (6H, m, H-1c, H-1c', 4 \times PhCH₂), 4.86-4.92 (2H, m, 2 \times PhCH₂), 5.14 (1H, s, H-1b), 5.20 (1H, s, H-1d'), 5.24 (1H, s, H-1d), 5.42-5.45 (2H, m, H-1a, H-2b), 6.69-6.72 (2H, m, 2 \times Ar-H), 6.79-6.83 (2H, m, 2 \times Ar-H), 7.10-7.37 (75H, m, 70 \times Ar-H); δ_C (100 MHz, CDCl₃) 12.0 (d, CH(CH₃)₂), 18.0 (q, CH(CH₃)₂), 21.1 (s, CH₃), 23.4 (s, COCH₃), 55.6 (q, OCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 62.9 (t, C-6c'), 67.1 (d, C-5a), 68.1 (d, C-5b), 69.1 (t, C-6a), 69.2 (t, C-6b), 69.3 (t, C-6d), 69.4 (t, C-6d'), 71.0 (d, C-2b), 71.4, 71.5, 71.8, 71.9, 72.0, 72.1, 72.2, 72.3, 72.3, 73.0, 73.7, 74.3, 74.5, 74.6, 74.7 (15 \times t, 15 \times PhCH₂), 72.8 (d, C-5d), 73.1 (d, C-

5d'), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.3 (d, C-5c'), 74.4 (d, C-4d), 74.5 (d, C-4d'), 74.5 (d, C-2c), 74.6 (d, C-2c'), 74.7 (d, C-4c), 74.8 (d, C-4c'), 75.1 (d, C-2d), 76.3 (d, C-2d'), 76.9 (d, C-3a), 78.8 (d, C-4a), 79.6 (d, C-4b), 79.7 (d, C-2c), 80.0 (d, C-2c'), 80.1 (d, C-3d), 81.4 (d, C-3d'), 96.7 (d, C-1a), 98.2 (d, C-1d), 98.4 (d, C-1d'), 98.6 (d, C-1c), 98.7 (d, C-1c'), 101.5 (d, C-1b), 114.3, 118.6, 126.9, 127.0, 127.1, 127.1, 127.2, 127.2, 127.3, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.6, 127.6, 127.7, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.2, 128.2, 128.2, 128.2, 128.3, 128.3, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.4, 129.6, 131.6, 133.8 (48 x d, 80 x Ar-C), 137.4, 138.0, 138.1, 138.3, 138.4, 138.5, 138.5, 138.6, 138.7, 138.8, 138.9, 139.0, 139.1, 139.2, 151.6, 155.1 (16 x s, 16 x Ar-C), 169.6, 170.5 (2 x s, 2 x C=O); HRMS (ES⁺) Calculated For C₁₇₀H₂₀₄NO₃₃Si₂ (MH⁺) 2845.3921. Found (MH⁺) 2845.3928.

***p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside**

3.22

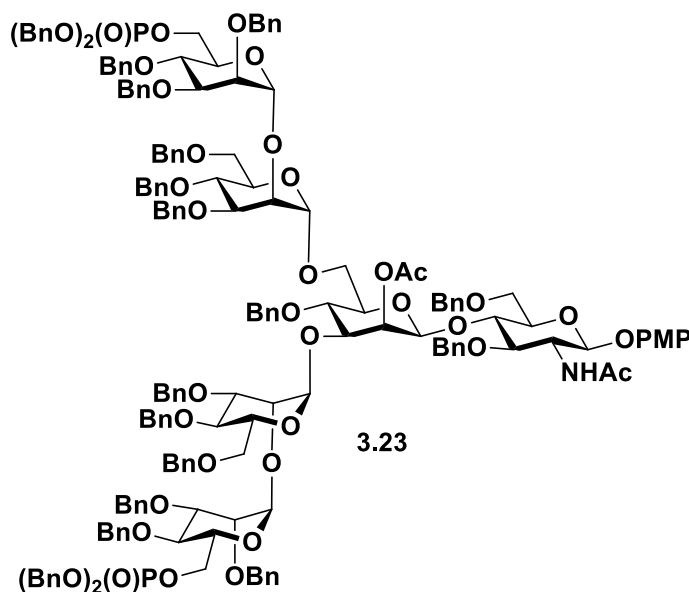


p-Methoxyphenyl 2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl-6-*O*-tri-*iso*-propylsilyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl-(1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside **3.21** (50 mg, 0.02 mmol) was dissolved in anhydrous DCM (5 mL) under an atmosphere of nitrogen in a flame-dried flask. The solution was cooled to 0 °C, and boron trifluoride diethyl etherate (22 μ L, 0.18 mmol) was added dropwise. The reaction was stirred at 0 °C for 1 h, after which time t.l.c. (petrol: ethyl acetate, 1:1) indicated the complete consumption of starting material (R_f 0.5) and the formation of a single product (R_f 0.3). The reaction mixture was diluted with DCM (10 mL), washed with sodium hydrogen carbonate (10 mL of a saturated aqueous solution), dried (Na₂SO₄), filtered, and concentrated *in vacuo*. The residue was purified by flash column

chromatography (petrol:ethyl acetate, 1:1) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl- α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside **3.22** (30 mg, 68%) as a white foam. $[\alpha]_{\text{D}}^{20} +15$ (*c*, 1.0 in CHCl₃); ν_{max} (KBr disk) 3428 (s, N-H stretch), 1746, 1739 (s, C=O) cm⁻¹; δ_{H} (400 MHz, CDCl₃) 1.60 (3H, s, COCH₃), 1.94 (3H, s, CH₃), 3.17-3.20 (1H, m, H-5b), 3.24-3.31 (1H, m, H-6b), 3.49-4.11 (31H, m, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H-4c', H-5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd'), 3.72 (3H, s, OCH₃), 4.23-4.29 (1H, m, H-3a), 4.21-4.65 (25H, m, H-2a, 24 x PhCH₂), 4.67 (1H, s, H-1c') 4.76- 4.89 (6H, m, 6 x PhCH₂), 4.77-4.89 (6H, m, 6 x PhCH₂), 4.95 (1H, s, H-1c), 5.01(1H, s, H-1d'), 5.04 (1H, s, H-1d), 5.16 (1H, s, H-1b), 5.31(1H, d, $J_{1,2}$ 8.0 Hz, H-1a), 5.38-5.40 (1H, m, H-2b), 6.69-6.72 (2H, m, 2 x Ar-H), 6.79-6.83 (2H, m, 2 x Ar-H), 7.10-7.37 (75H, m, 70 x Ar-H); δ_{C} (100 MHz, CDCl₃) 21.0 (s, CH₃), 23.2 (s, COCH₃), 55.6 (q, OCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 62.9 (t, C-6c'), 67.1 (d, C-5a), 68.1 (d, C-5b), 69.1 (t, C-6a), 69.2 (t, C-6b), 69.3 (t, C-6d), 69.4 (t, C-6d'), 71.0 (d, C-2b), 71.4, 71.5, 71.8, 71.9, 72.0, 72.1, 72.2, 72.3, 72.3, 73.0, 73.7, 74.3, 74.5, 74.6, 74.7 (15 x t, 15 x PhCH₂), 72.8 (d, C-5d), 73.1 (d, C-5d'), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.3 (d, C-5c'), 74.4 (d, C-4d), 74.5 (d, C-4d'), 74.5 (d, C-2c), 74.6 (d, C-2c'), 74.7 (d, C-4c), 74.8 (d, C-4c'), 75.1 (d, C-2d), 76.3 (d, C-2d'), 76.9 (d, C-3a), 78.8 (d, C-4a), 79.6 (d, C-4b), 79.7 (d, C-2c), 80.0 (d, C-2c'), 80.1 (d, C-3d), 81.4 (d, C-3d'), 97.1 (d, C-1a), 98.7 (d, C-1d), 98.8 (d, C-1d'), 99.8 (d, C-1c), 100.0 (d, C-1c'), 101.5 (d, C-1b), 114.3, 118.6,

126.9, 127.0, 127.1, 127.1, 127.2, 127.2, 127.3, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.6, 127.6, 127.7, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.2, 128.2, 128.2, 128.2, 128.3, 128.3, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 129.4, 129.6, 131.6, 133.8 (48 x d, 80 x Ar-C), 137.4, 138.0, 138.1, 138.3, 138.4, 138.5, 138.5, 138.6, 138.7, 138.8, 138.9, 139.0, 139.1, 139.2, 151.6, 155.1 (16 x s, 16 x Ar-C), 169.6, 170.5 (2 x s, 2 x C=O); HRMS (ES⁺) Calculated For C₁₅₂H₁₆₄NO₃₃ (MH⁺) 2532.1219. Found (MH⁺) 2532.1242.

***p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl -6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl -6-*O*-dibenzoyloxyphosphoryl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl- (1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside 3.23**



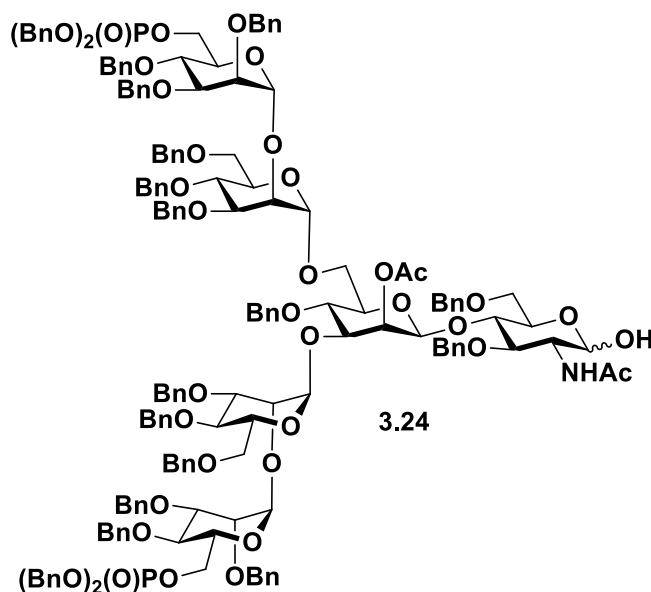
p-Methoxyphenyl 2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside

3.22 (13 mg, 0.005 mmol) and 1*H*-tetrazole (0.08 mL of a 0.45 M solution in acetonitrile) were added to a flame-dried flask and dissolved in DCM (0.50 mL). The solution was stirred at rt 1h under nitrogen atmosphere. Dibenzyl N, N-diisopropyl phosphoramidate (0.014 mL, 0.042 mmol) was added, and the reaction stirred for 18 h. After this time t.l.c. (MeOH:DCM, 1:50) indicated complete consumption of alcohol starting material (R_f 0.30) and formation of a major product (R_f 0.60). The reaction mixture was cooled to -78 °C and *m*-chloroperbenzoic acid (0.01 g, 0.02 mmol) was added. The mixture was stirred for 2 h and then warmed to rt, after which time t.l.c. (MeOH:DCM, 1:50) indicated complete consumption of the phosphine intermediate (R_f 0.60) and formation of a major product (R_f 0.25). The reaction was quenched by addition of sodium bisulfite (5 mL of a 10% w/v aqueous solution), stirred for 10 min, and extracted with DCM (10 mL). The organic extracts were washed with sodium hydrogen carbonate (5 mL of a saturated aqueous solution) and brine (5 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (MeOH:DCM, 1:50) to afford *p*-Methoxyphenyl 2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl -6-*O*-dibenzyloxyphosphoryl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl -6-*O*-dibenzyloxyphosphoryl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-

mannopyranosyl- (1→3)]-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy -β-D-glucopyranoside **3.23** (0.015 g, 94% over two steps) as a white foam. $[\alpha]_{\text{D}}^{20} +12$ (*c*, 1.0 in CHCl₃); ν_{max} (KBr disk) 3430 (s, N-H stretch), 1744, 1740 (s, C=O) cm⁻¹; δ_{H} (400 MHz, CDCl₃) 1.60 (3H, s, COCH₃), 1.96 (3H, s, CH₃), 3.36-3.48 (2H, m, H-5b, H-6b), 3.60-4.15 (31H, m, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H-4c', H-5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd'), 3.72 (3H, s, OCH₃), 4.23-4.29 (1H, m, H-3a), 4.21-4.62 (16H, m, H-2a, 16 x PhCH₂), 4.01-5.04 (20H, m, H-1c, H-1c', H-1d, H-1d', 16 x PhCH₂), 5.32 (1H, s, H-1b), 5.36 (1H, d, $J_{1,2}$ 8.0 Hz, H-1a), 5.58-5.59 (1H, m, H-2b), 6.69-6.72 (2H, m, 2 x Ar-H), 6.79-6.83 (2H, m, 2 x Ar-H), 7.10-7.37 (95H, m, 70 x Ar-H); δ_{C} (100 MHz, CDCl₃) 21.0 (s, CH₃), 23.2 (s, COCH₃), 55.6 (q, OCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 62.9 (t, C-6c'), 67.1 (d, C-5a), 68.1 (d, C-5b), 69.1 (t, C-6a), 69.2 (t, C-6b), 69.3 (t, C-6d), 69.4 (t, C-6d'), 71.0 (d, C-2b), 71.4, 71.5, 71.8, 71.9, 72.0, 72.1, 72.2, 72.3, 72.3, 73.0, 73.7, 74.3, 74.5, 74.6, 74.7 (15 x t, 15 x PhCH₂), 72.8 (d, C-5d), 73.1 (d, C-5d'), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.3 (d, C-5c'), 74.4 (d, C-4d), 74.5 (d, C-4d'), 74.5 (d, C-2c), 74.6 (d, C-2c'), 74.7 (d, C-4c), 74.8 (d, C-4c'), 75.1 (d, C-2d), 76.3 (d, C-2d'), 76.9 (d, C-3a), 78.8 (d, C-4a), 79.6 (d, C-4b), 79.7 (d, C-2c), 80.0 (d, C-2c'), 80.1 (d, C-3d), 81.4 (d, C-3d'), 97.1 (d, C-1a), 98.7 (d, C-1d), 99.1 (d, C-1d'), 99.3 (d, C-1c), 99.8 (d, C-1c'), 101.2 (d, C-1b), 114.3, 118.6, 126.8, 126.9, 126.9, 127.0, 127.1, 127.1, 127.2, 127.2, 127.2, 127.2, 127.3, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.5, 127.5, 127.6, 127.6, 127.6, 127.6, 127.6, 127.7, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 127.9, 127.9, 127.9, 127.9, 128.0, 128.0, 128.0, 128.1, 128.1, 128.2, 128.2, 128.2, 128.3, 128.3, 128.3, 128.3, 128.3, 128.3, 128.4, 128.4, 128.4, 128.5, 128.5, 128.5,

128.6, 128.6 (59 x d, 100 x Ar-C), 135.7, 135.8, 135.8, 135.9, 135.9, 135.9, 138.0, 138.1, 138.2, 138.2, 138.3, 138.3, 138.4, 138.5, 138.6, 138.7, 138.8, 139.0, 139.2, 151.7, 154.9 (21 x s, 21 x Ar-C), 169.6, 170.5 (2 x s, 2 x C=O); δ_P (162 MHz, $CDCl_3$) -1.25, -1.54; HRMS (ES^+) Calculated For $C_{180}H_{191}NO_{39}P_2$ ($M+2H$) $^{2+}$ 1526.6246. Found $[M+2H]^{2+}$ 1526.6270.

2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl -6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl -6-*O*-dibenzoyloxyphosphoryl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside 3.24

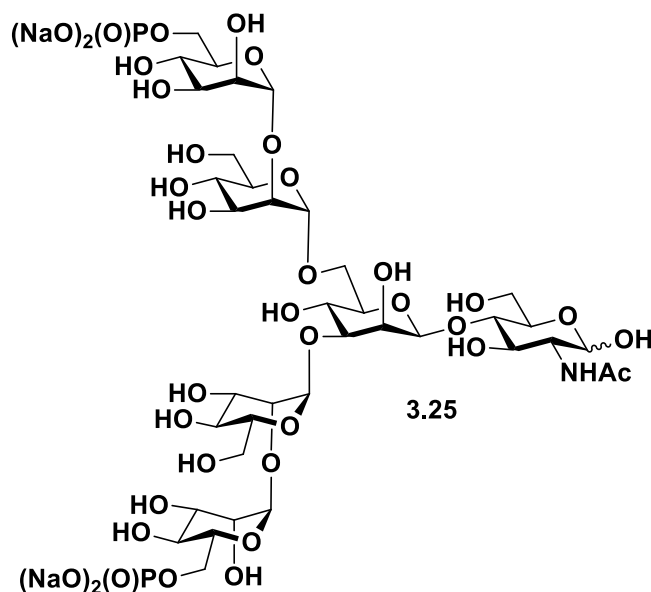


p-Methoxyphenyl 2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl -6-*O*-dibenzoyloxyphosphoryl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl -6-*O*-dibenzoyloxyphosphoryl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-

benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside **3.23** (15 mg, 0.005 mmol) was suspended in a mixture of acetonitrile (0.8 mL) and water (0.21 mL). Ceric ammonium nitrate (14 mg, 0.025 mmol) was added, and the reaction mixture stirred at rt. After 1 h, t.l.c. (MeOH:DCM, 1:50) indicated complete consumption of starting material (R_f 0.25) and formation of a single product (R_f 0.15). The reaction mixture was diluted with DCM (10 mL) and washed with sodium hydrogen carbonate (2 x 5 mL of a saturated aqueous solution), sodium thiosulfate (2 x 5 mL of a 5% w/v aqueous solution), EDTA (2 x 5 mL of a 0.1 M aqueous solution) and water (5 mL). The organic extracts were dried (Na_2SO_4), filtered, concentrated *in vacuo*, and the residue purified by flash column chromatography (MeOH:DCM, 1:50) to afford 2,3,4-tri-*O*-benzyl - α -D-mannopyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl -6-*O*-dibenzyloxyphosphoryl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl -6-*O*-dibenzyloxyphosphoryl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside **3.24** (11.6 mg, 80%) as a pale yellow foam. ν_{max} (KBr disk) 3430 (s, N-H stretch), 1740, 1735 (s, C=O) cm^{-1} ; δ_{H} (400 MHz, CDCl_3) 1.65 (3H, s, COCH_3), 1.97 (3H, s, CH_3), 3.21-4.15 (33H, m, H-5b, H-6b, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H-4c', H-5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd'), 3.72 (3H, s, OCH_3), 4.23-4.29 (1H, m, H-3a), 4.21-4.62 (16H, m, H-2a, 16 x PhCH_2), 4.01-5.04 (21H, m, H-1a, H-1c, H-1c', H-1d, H-1d', 16 x PhCH_2), 5.27 (1H, s,

H-1b), 5.53 (1H, brs, H-2b), 7.10-7.37 (95H, m, 70 x Ar-H) δ_C (100 MHz, CDCl₃) 21.0 (s, CH₃), 23.2 (s, COCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 62.9 (t, C-6c'), 67.1 (d, C-5a), 68.1 (d, C-5b), 69.1 (t, C-6a), 69.2 (t, C-6b), 69.3 (t, C-6d), 69.4 (t, C-6d'), 71.0 (d, C-2b), 71.4, 71.5, 71.8, 71.9, 72.0, 72.1, 72.2, 72.3, 72.3, 73.0, 73.7, 74.3, 74.5, 74.6, 74.7 (15 x t, 15 x PhCH₂), 72.8 (d, C-5d), 73.1 (d, C-5d'), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.3 (d, C-5c'), 74.4 (d, C-4d), 74.5 (d, C-4d'), 74.5 (d, C-2c), 74.6 (d, C-2c'), 74.7 (d, C-4c), 74.8 (d, C-4c'), 75.1 (d, C-2d), 76.3 (d, C-2d'), 76.9 (d, C-3a), 78.8 (d, C-4a), 79.6 (d, C-4b), 79.7 (d, C-2c), 80.0 (d, C-2c'), 80.1 (d, C-3d), 81.4 (d, C-3d'), 96.8 (d, C-1a), 99.3 (d, C-1d), 99.4 (d, C-1d'), 99.5 (d, C-1c), 99.6 (d, C-1c'), 101.2 (d, C-1b), 126.8, 126.9, 126.9, 127.0, 127.1, 127.1, 127.2, 127.2, 127.2, 127.2, 127.3, 127.3, 127.3, 127.4, 127.4, 127.4, 127.5, 127.5, 127.5, 127.5, 127.6, 127.6, 127.6, 127.6, 127.6, 127.7, 127.7, 127.7, 127.8, 127.8, 127.8, 127.9, 127.9, 127.9, 127.9, 127.9, 128.0, 128.0, 128.0, 128.1, 128.1, 128.2, 128.2, 128.2, 128.2, 128.3, 128.3, 128.3, 128.3, 128.3, 128.3, 128.4, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.6 (57 x d, 95 x Ar-C), 135.7, 135.8, 135.8, 135.9, 135.9, 135.9, 138.0, 138.1, 138.1, 138.2, 138.2, 138.3, 138.3, 138.4, 138.5, 138.6, 138.7, 138.8, 139.2 (19 x s, 19 x Ar-C), 169.7, 171.3 (2 x s, 2 x C=O); δ_P (162 MHz, CDCl₃) -1.37, -1.50 HRMS (ES⁺) Calculated For C₁₇₃H₁₈₅NO₃₈P₂ [M+2H]²⁺ 1474.1053. Found (M+2H)²⁺ 1474.1068.

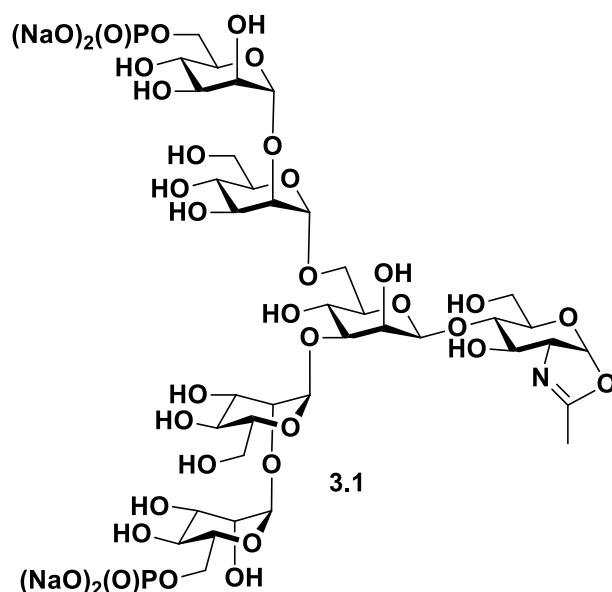
6-O-Phosphate - α -D-manno pyranosyl- (1→2) - α -D-mannopyranosyl- (1→6)- [6-O-Phosphate - α -D-manno pyranosyl- (1→2)- α -D-mannopyranosyl- (1→3)]- β -D-mannopyranosyl-(1→4))-2-acetamido-2-deoxy-D-glucopyranose 3.25



2,3,4-tri-*O*-benzyl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl -6-*O*-dibenzyloxyphosphoryl- α -D-mannopyranosyl- (1 \rightarrow 6)-2-*O*-acetyl-4-*O*-benzyl-[2,3,4-tri-*O*-benzyl -6-*O*-dibenzyloxyphosphoryl - α -D-manno pyranosyl- (1 \rightarrow 2)- 3,4,6- tri-*O*-benzyl- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy - β -D-glucopyranoside **3.24** (30 mg, 0.01 mmol) in THF (20 mL) was added to NH_3 (*l*) (20 mL) at -33° C. The minimum amount of sodium for the mixture to turn deep blue was added to the stirred mixture. After 30 min MeOH (4 mL) was added, and reaction mixture was stirred for a futher 1 h, warmed to rt, and the solvent was removed *in vacuo*. Gel filtration of the crude residue on a Sephadex G-10 column (eluting with 0.01% NH_3) afforded 6-*O*-Phosphate - α -D-manno pyranosyl- (1 \rightarrow 2) - α -D-mannopyranosyl- (1 \rightarrow 6)- [6-*O*-Phosphate - α -D-manno pyranosyl- (1 \rightarrow 2)- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4))-2-acetamido-2-deoxy-D-glucopyranose **3.25** (10.2 mg, 83%), as a white foam as a mixture of α : β anomers. δ_{H}

(400 MHz, CDCl₃) 1.98 (3H, s, CH₃), 3.11-4.19 (35H, m, H-5b, H-6b, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H-4c', H-5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd', H-3a, H-2a), 4.92-5.09 (5H, m, H-1a, H-1c, H-1c', H-1d, H-1d'), 5.13 (1H, bs, H-1a), 5.31 (1H, brs, H-1b); δ_C (100 MHz, CDCl₃) 23.2 (s, COCH₃), 55.7 (d, C-2a), 62.5 (t, C-6c), 62.9 (t, C-6c'), 67.1 (d, C-5a), 68.1 (d, C-5b), 69.1 (t, C-6a), 69.2 (t, C-6b), 69.3 (t, C-6d), 69.4 (t, C-6d'), 71.0 (d, C-2b), 72.8 (d, C-5d), 73.1 (d, C-5d'), 73.2 (d, C-3b), 74.3 (d, C-5c), 74.3 (d, C-5c'), 74.4 (d, C-4d), 74.5 (d, C-4d'), 74.5 (d, C-2c), 74.6 (d, C-2c'), 74.7 (d, C-4c), 74.8 (d, C-4c'), 75.1 (d, C-2d), 76.3 (d, C-2d'), 76.9 (d, C-3a), 78.8 (d, C-4a), 79.6 (d, C-4b), 79.7 (d, C-2c), 80.0 (d, C-2c'), 80.1 (d, C-3d), 81.4 (d, C-3d'), 96.7 (d, C-1a), 99.3 (d, C-1d), 99.4 (d, C-1d'), 99.5 (d, C-1c), 99.6 (d, C-1c'), 101.2 (d, C-1b), 171.3 (s, C=O); δ_P (162 MHz, CDCl₃) 3.91, 4.13; HRMS (ES⁺) Calculated For C₃₈H₆₈NO₃₇P₂ (MH⁺) 1192.2945. Found (MH⁺) 1192.2925.

2-Methyl [6-O-Phosphate - α -D-manno pyranosyl- (1→2)- α -D-mannopyranosyl- (1→6)- [6-O-Phosphate - α -D-manno pyranosyl- (1→2)- α -D-mannopyranosyl- (1→3)]- β -D-mannopyranosyl-(1→4))-2-acetamido-1,2-deoxy-D-glucopyrano]-[2,1-d]-oxazoline 3.1



6-*O*-Phosphate - α -D-manno pyranosyl- (1 \rightarrow 2) - α -D-mannopyranosyl- (1 \rightarrow 6)- [6-*O*-Phosphate - α -D-manno pyranosyl- (1 \rightarrow 2)- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4))-2-acetamido-2-deoxy-D-glucopyranose **3.25** (4 mg, 0.0034 mmol) and triethylamine (4.3 μ L, 0.03 mmol) were dissolved in D₂O (37 μ L) and the resulting solution was cooled to 0 °C. DMC (1.7 mg, 0.01 mmol) was added to the solution and the mixture was stirred for 30 min at the same temperature. Gel filtration of the residue on a Sephadex G-10 column eluted with 0.01% NH₃ afforded 2-Methyl [6-*O*-Phosphate - α -D-manno pyranosyl- (1 \rightarrow 2)- α -D-mannopyranosyl- (1 \rightarrow 6)- [6-*O*-Phosphate - α -D-manno pyranosyl- (1 \rightarrow 2)- α -D-mannopyranosyl- (1 \rightarrow 3)]- β -D-mannopyranosyl-(1 \rightarrow 4))-2-acetamido-1,2-deoxy-D-glucopyrano]-[2,1-*d*]-oxazoline **3.1** (1.8 mg, 95%) as a white foam. δ_{H} (400 MHz, CDCl₃) 1.98 (3H, s, CH₃), 3.11-4.19 (35H, m, H-5b, H-6b, H-4a, H-5a, H-6a, H-6'a, H-3b, H-4b, H-6'b, H-2c, H-3c, H-4c, H-5c, H-6c, H-6'c, H-2d, H-3d, H-4d, H-5d, H-6d, H-6'd, H-2c', H-3c', H4c', H5c', H-6c', H-6'c', H-2d', H-3d', H-4d', H-5d', H-6d', H-6'd', H-3a, H-2a), 4.92-5.09 (5H, m, H-1a, H-1c, H-1c', H-

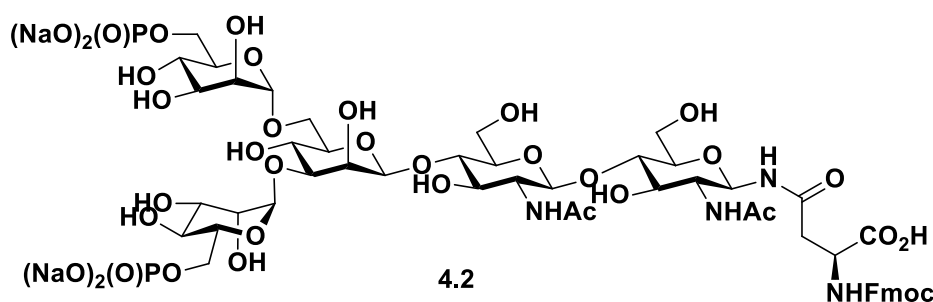
1d, H-1d'), 5.31 (1H, brs, H-1b) 6.08 (1H, d, $J_{1,2}$ 7.3 Hz, H-1a); δ_P (162 MHz, $CDCl_3$) 4.46; HRMS (ES^+) Calculated For $C_{38}H_{67}NO_{37}P_2$ $[M-H_2O+H]^+$ 1192.29. Found $(M-H_2O+H)^+$ 1192.29.

5.4 Experimental for chapter 4

General procedure for the enzymatic glycosylation of acceptor with oxazoline donor

Endohexosaminidase-catalysed glycosylations were monitored by HPLC using a Dionex 1 HPLC instrument using Chromeleon software connected to a Dionex 1 variable wavelength detector at 254 nm wavelength. Analytical HPLC (Jupitor 5 μ C-18 column, 250 x 4.6 mm) was used to monitor the reactions, with 2 μ L aliquots taken at appropriate time intervals. The column was eluted with 20% MeCN/ H_2O . The yield was determined by integration of the product and acceptor peaks.

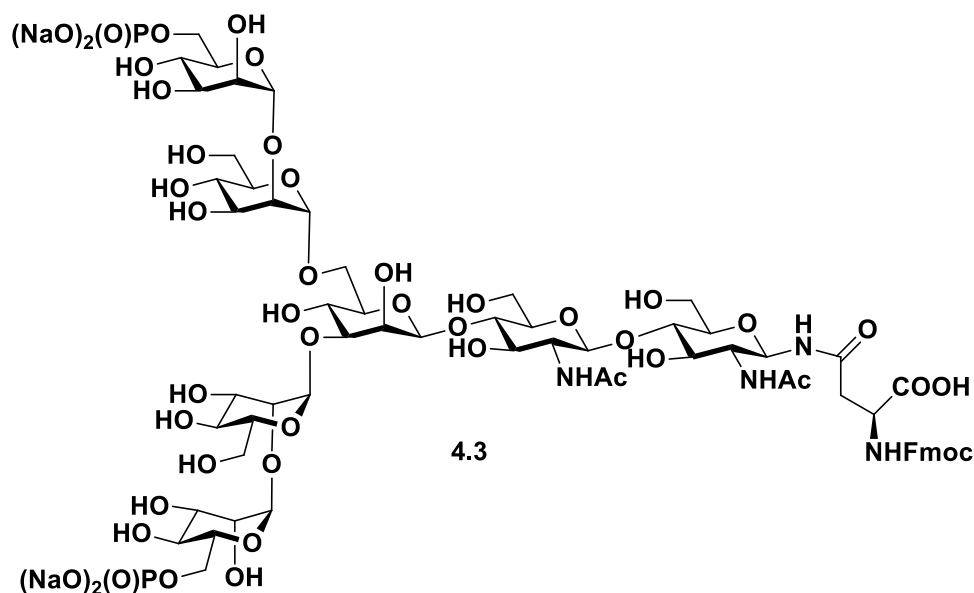
Glycosylation of the tetrasaccharide oxazoline donor 2.1 with Fmoc-Asn(GlcNAc)-OH 4.1 with Endo A:



A solution of the tetrasaccharide oxazoline **13** (0.762 mg, 0.9 μ mol) and Fmoc-Asn(GlcNAc)-OH **14** (0.058 mg, 0.1 μ mol) was incubated with 2 mU of Endo A in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 23 $^{\circ}C$. After 2 h, RP-HPLC indicated

the formation of a new product, which was then purified directly by RP-HPLC to give **15**: (71%, HPLC yield based on acceptor) as a white powder (0.88 mg); analytical HPLC: $t_R = 9.71$ min; ESI-MS: calculated for $C_{53}H_{77}N_4O_{36}P_2$: 1408.3826. Found: 1408.3880 (M+H)⁺; $C_{53}H_{76}N_4NaO_{36}P_2$: 1430.3646. Found: 1429.3683 (M+Na).

Glycosylation of the hexasaccharide oxazoline donor **3.1 with Fmoc-Asn(GlcNAc)-OH **4.1** with Endo A:**



A solution of the hexasaccharide oxazoline **3.1** (0.34 mg, 0.30 μ mol) and Fmoc-Asn(GlcNAc)-OH **4.1** (0.06 mg, 0.10 μ mol) was incubated with 1 μ g of Endo-A in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 37 °C. After 2 h, RP-HPLC indicated the formation of a new product, which was then purified directly by RP-HPLC to give **4.3** (12%, HPLC yield based on acceptor); $t_R = 10.6$ min; ESI-MS: calculated for $C_{50}H_{87}N_4O_{44}P_2$ (M-Fmoc+H)⁺: 1507.4932. Found: 1507.4993 (M-Fmoc+H)⁺

Glycosylation of the hexasaccharide oxazoline donor with Fmoc-Asn(GlcNAc)-OH using WT Endo M:

A solution of the hexasaccharide oxazoline **3.1** (0.66 mg, 0.50 μ mol) and Fmoc-Asn(GlcNAc)-OH **4.1** (0.06 mg, 0.10 μ mol) was incubated with 1 μ g of WT Endo-M in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 37 °C. After 2 h, RP-HPLC indicated the formation of a new product, which was then purified directly by RP-HPLC to give **4.3** (6%, HPLC yield based on acceptor); t_R = 10.6 min; ESI-MS: calculated for $C_{50}H_{87}N_4O_{44}P_2$ (M-Fmoc+H)⁺: 1509.42. Found: 1509.42(M-Fmoc+H)⁺

Glycosylation of the hexasaccharide oxazoline donor with Fmoc-Asn(GlcNAc)-OH using the N175Q Endo M mutant:

A solution of the hexasaccharide oxazoline **3.1** (0.66 mg, 0.50 μ mol) and Fmoc-Asn(GlcNAc)-OH **4.1** (0.06 mg, 0.10 μ mol) was incubated with 1 μ g of Endo M N175Q in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 37 °C. After 2 h, RP-HPLC indicated the formation of a new product, which was then purified directly by RP-HPLC to give **4.3** (8%, HPLC yield based on acceptor); t_R = 10.6 min; ESI-MS: calculated for $C_{50}H_{87}N_4O_{44}P_2$ (M-Fmoc+H)⁺: 1509.42. Found: 1509.42 (M-Fmoc+H)⁺.

Glycosylation of dRNase B 4.4 with 2.1

A solution of the tetrasaccharide oxazoline **2.1** (0.229 mg, 0.27 μ mol) and dRNase B **4.4** (0.348 mg, 0.03 μ mol) was incubated with 2 mU of Endo-A in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 23 °C for 2h to give (M6P)₂RNase **4.5**. $C_{34}H_{61}N_2O_{32}P_2$ -RNase B: 14735.4633. Found: 14735.4688 (M+H)⁺.

Glycosylation of dRNase B 4.4 with 3.1

A solution of the tetrasaccharide oxazoline **3.1** (0.322 mg, 0.27 μ mol) and dRNase B **4.4** (0.348 mg, 0.03 μ mol) was incubated with 2 mU of Endo-A in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 23 °C for 2h to give (M6P)₂RNase **4.6**. HRMS (ES⁺) Calculated For C₃₈H₆₈NO₃₇P₂ (MH⁺) 1192.29. Found (MH⁺) 1192.27.

Glycosylation of de-glycosylated fabrazyme 4.7 with 2.1

Fabrazyme (5.0 mg) was deglycosylated with wild type Endo A (50 μ g of Endo A) in 5 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 37 °C for 10 h. After Endo A mediated hydrolysis was complete the de-glycosylated Fabrazyme (dFab) was purified using RP-HPLC to give pure dFab **4.7**.

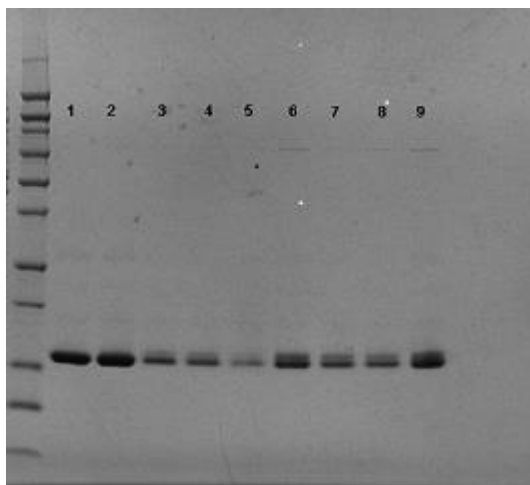
A solution of the tetrasaccharide oxazoline **2.1** (0.24 mg, 0.28 μ mol) and dFab **4.7** (0.21 mg, 0.005 μ mol) was incubated with 1 μ g of Endo-A in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 37 °C for 2h to give double glycosylation of dFab with the addition of two tetrasaccharide units – M6P₂Fab. HRMS: calculated for (2[M6P-tetra]-dFab+2H⁺): 48218.30; Found: 48218.30.

Glycosylation of de-glycosylated fabrazyme 4.7 with 3.1

A solution of the hexasaccharide oxazoline **3.1** (0.35 mg, 0.30 μ mol) and dFab **4.7** (0.21 mg, 0.005 μ mol) was incubated with 1 μ g of Endo-A in 20 μ L of sodium phosphate buffer (100 mM, pH 6.5) at 37 °C for 2h to give double glycosylation of dFab with the addition of two hexasaccharide units – M6P₂Fab. HRMS: 49210.30.

Appendix

A.1 SDS PAGE for enzymatic glycosylation for dRNase B



Lane 1- 0min, Lane 2-15min, Lane 3-30min, Lane 4-1h, Lane 5-2h, Lane 6-4h, Lane 7-8h, Lane 8-24h, Lane 9-reaction mixture.

A.2 Immunocytochemical detection of M6P-RNase in HepG2 cells

HepG2 thawing and culture and maintenance

Human hepatocellular carcinoma (HepG2) cells were sourced from the laboratory of Prof John Evans (University of Otago, Christchurch School of Medicine). All cell culture was performed in a Class II Biohazard hood using sterile reagents and consumables.

1.5×10^6 HEPG2 cells in 10% cell culture grade DMSO/growth medium (See page 209) were gently thawed from liquid nitrogen store in a 37°C water bath. When the last ice crystals were evident, the cells were gently transferred into 5 mL of fresh pre-warmed (37 °C) growth medium. Cells were spun at 1,000 rpm for 5 minutes at room temperature. The supernatant was removed by aspiration and the pellet gently re-suspended in 15 mL of growth media and transferred into a T75 flask (Corning) and cultured at 37 °C/5% CO₂

in a static tissue culture incubator (Sanyo). Growth media was changed by aspiration and replacement of 15 mL of fresh, pre-warmed growth media every 2-3 days. Cells were cultured until reaching a confluence of around 70-80%.

HepG2 passaging

On reaching 70-80% confluence, cells were trypsinized to liberate the cells from the bottom of the flask. To achieve this, HepG2 cells were washed with 5 mL of sterile phosphate buffered saline (PBS, Life Technologies), with PBS aspirated, washed again with another 5 mL of PBS and removed. Five mL of 0.05% Trypsin/EDTA (Life Technologies) was added to the plate and incubated at 37 °C/5% CO₂ for 5 minutes. After 5 minutes, the flask was inspected under the light microscope to confirm detachment and the reaction quenched with addition of 10 mL of fresh, pre-warmed growth media. Cells were counted using either a hemocytometer under a light microscope, or using a Countess Automated Cell Counter (Life Technologies). Typically cells were split 1/3 across new T75 flasks and propagated as outlined in section 1.1. Specific cell numbers were also taken forward for immunocytochemical experiments indicated in section 1.3.

Immunocytochemistry

HepG2 cells were plated into each well of a LabTek 4 well Chamber Slide (Nunc) at 75,000 cells/well. Cells were incubated at 37 °C/5% CO₂ for 2 days. After 2 days, each well was washed twice with 1 mL of pre-warmed (37 °C) PBS/well. Cells were incubated in the presence of either 1 µM M6P-RNase, µM deglycosylated RNase or media only for 1-2 hours at 37 °C/5% CO₂. Incubation media altered from standard growth media, with

FBS removed (MEM, 100U penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin). Extra wells were also prepared for immunocytochemistry controls.

After incubation, media was aspirated from the chamber slide wells and washed twice with 1mL of PBS per wash. The cells were then fixed by addition of 1 mL 4% paraformaldehyde in PBS to each well and incubated at 4 °C for 30 min. At the conclusion of 30 minutes, the paraformaldehyde was aspirated and the wells washed twice with 1mL of PBS per wash with a 5 minute incubation period for each wash. The cells were then permeabilized with 0.5 mL 80% methanol/PBS at -20 °C for 15 minutes. After 15 minutes, the cells were again washed with two 5 minute PBS washes. The cells were then blocked with 0.5 mL 3% non-fat dried milk/1% bovine serum albumin in PBS for 30 min at room temperature. After 30 minutes, the cells were again washed with two 5 minute PBS washes. Appropriate wells were co-incubated with 1:10,000 mouse anti-cation independent mannose-6-phosphate receptor (CI-MPR, Abcam) and 1:10,000 rabbit anti-RNase A (Abcam) in 0.1% BSA/PBS (0.5 mL/well) and incubated for 1 hour at room temperature. After 1 hour, the cells were again washed with two 5 minute PBS washes. Both 1:400 goat anti-mouse IgG-Alexa 488 (Life Technologies) and 1:400 goat anti-rabbit IgG-Alexa 568 (Life Technologies) in 0.1% BSA/PBS were added to the wells (0.5 mL/well) and incubated for 1 hour at room temperature in the dark. After 1 hour, the cells were again washed with two 5 minute PBS washes. Cells were then incubated with 0.5 mL of 300 nM 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) in PBS and incubated for 1 minute at room temperature in the dark to stain cell nuclei. After 1 minute, the cells were again washed with two 5 minute PBS washes.

Chambers were then removed and slides mounted with mounting media (Dako, Australia) and coverslipped. Imaging was performed using a Leica SP5 confocal laser scanning microscope (Wetzlar, Germany) fitted with a 63X NA1.3 glycerol immersion lens and excitation at 405, 488, 561 nm (violet, blue, and green respectively) and LAS software. Fluorescence on the SP5 system collected in user-defined wavelength bands. Fluorescence was emission monitored between 415 and 485 nm using 405 nm excitation for DAPI, emission between 555 and 625 nm and 488 nm excitation for CI-MPR, emission between 695 and 765 nm with excitation for RNase fluorescence at 561 nm. To reduce cross-talk the different fluorophores, images were collected using sequential line scanning with only a single excitation wavelength and the corresponding fluorescence collected at any one time. At least three images were captured per well with duplicate wells per treatment.

Control wells were included which did not receive either primary or secondary antibody or both, to control for background from non-specific binding/background fluorescence. Bleed through was also evaluated for each fluorophore.

A.3 Immunoprecipitation

Binding of CI-MPR to M6P-RNase was also demonstrated. Prior to immunoprecipitation experiments, HepG2 cell lysate was prepared in order to capture CI-MPR.

Preparation of HepG2 cell lysate

HepG2 cells were cultured to 80% confluence as described in sections 1.1. and 1.2 in a T75 flask. Media was aspirated and cells washed twice with 2 mL 1x cold PBS. PBS was

aspirated and 1 mL of radioimmunoprecipitation (RIPA) buffer (Sigma) containing protease inhibitor cocktail (Roche) was added to the flask which was then scraped with a rubber policeman on ice. The contents of the flask were then transferred to a 2 mL microcentrifuge tube on ice. The sample was sonicated for 4 x 30 second pulses (Hielscher Ultrasonic Processor, 35% amplitude, cycle @ 0.3 = 30% power) while on ice using a micro-tip. The sample was then incubated on ice for 30 minutes, vortexing every 10 min. After 30 minutes the lysate was spun at 14,000 x g for 15 min at 4°C. The supernatant, the total protein fraction was retained. Protein was quantitated by Bicinchoninic Acid (BCA) Assay according to manufacturer's instructions (Pierce). Lysate was stored at -20 °C until required.

Coupling of CI-MPR antibody to Protein A resin

One hundred µL of Protein A sepharose (GE) was added to each of 3 microcentrifuge tubes. The tubes were spun at 1,000 x g for 2 min at 4°C and the supernatant removed. The pellet was resuspended in 500 µL RIPA buffer and re-spun. This RIPA wash was repeated one more time. The supernatant was discarded and the pellet resuspended in 99 µL RIPA on ice and 1 µL of 1 mg/ml mouse anti-CI-MPR (Abcam) antibody added to each tube. The tubes were incubated for 2 hours at 4 °C on a tube mixer. After 2 hours, the tubes were centrifuged at 2,000 x g for 2 minutes at 4 °C and the supernatant discarded. One mL of RIPA buffer was added with gentle agitation and centrifuged at 3,000 x g for 2 min at 4 °C. This wash step was repeated twice more.

Addition of HPEG2 cell lysate to anti-CI-MPR conjugated Protein A to capture CI-MPR

Fifty µg of HepG2 lysate prepared in section 1.4.1 was diluted in RIPA buffer to a final volume of 100 µL and added to anti-CI-MPR conjugated resin prepared in section 1.4.2 (9.2 µL lysate (5.43 mg/ml) + 90.8 µL RIPA buffer). The lysate-beads/antibody mixtures were incubated 4 °C overnight on tube mixer. After incubation the tubes were centrifuged at 2,000 x g for 2 minutes at 4 °C, the supernatant removed and the pellet washed with 1 mL RIPA buffer three times to remove non-specific binding. During removal of supernatant from the last wash, efforts were made to remove as much wash buffer as possible from the pellet.

Incubation of RNases with immobilized CI-MPR

The CI-MPR-conjugated resin prepared in section 1.4.2 was incubated with either of the three samples below at a final amount of 2 µg in RIPA buffer to a final volume of 100 µL:

- a. M6P RNase
- b. deglycosylated RNase (dRNase)
- c. No RNase

The tubes were incubated at 4 °C overnight on tube mixer. After incubation, tubes were centrifuged at 2,000 x g for 2 minutes at 4°C. Tubes were washed with 1 mL RIPA buffer 5x to remove non-specifically bound protein. Efforts were made to remove as much wash buffer as possible from the pellet.

SDS-PAGE and Western Blotting to detect RNase capture by CI-MPR

Protein was eluted from the washed pellet in section 1.4.3 by heating the pellet at 50°C for 10 minutes in 7.5 µL 4x LDS buffer (Novex gel kit buffer, Life Technologies) + 22.5 µL RIPA buffer + 5 µL 10x reducing buffer (Novex gel kit buffer, Life Technologies). Tubes were spun at 2,000 x g for 2 minutes at room temperature and the supernatant retained (Elution 1). The elution steps above were repeated the elution retained (Elution 2).

Positive controls were prepared to confirm RNase molecular weight and staining where M6P-RNase and dRNase were diluted to 25 ng in RIPA buffer, 4x LDS buffer and 10x reducing buffer.

All samples were incubated at 100 °C for 5 min and loaded onto a 12% Novex Bolt Bis-Tris Plus Gel (Life Technologies). The gel was resolved in 1x 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Life Technologies) in a Bolt electrophoresis rig (Life Technologies) at 165V for 40 minutes. The gel was removed from the cassette and equilibrated in transfer buffer (48 mM Tris base, 39 mM glycine, 20% methanol, pH 9.2). Nitrocellulose membrane (Bio-Rad) was also equilibrated in transfer buffer for 15 minutes. Following equilibration of both gel and membrane, transfer of protein from the SDS-PAGE gel to the nitrocellulose membrane was conducted using a Bio-Rad Semi Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 25V for 15 minutes according to the manufacturer's instructions. At the conclusion of transfer, membrane washed for two 5 minute washes in 20 mL of reverse osmosis (RO) water with agitation. Water was removed from the membrane and the membrane blocked overnight at 4 °C in 10 mL of blocking buffer from an Anti-Rabbit

WesternBreeze Chromogenic Kit (Life Technologies). After incubation, the membrane washed for two 5 minute washes in 20 mL of RO water with agitation. Water was removed and 2 mL of 1:1000 rabbit anti-RNase (Abcam) diluted in blocking buffer was added to the membrane and incubated at room temperature for 1 hour. After 1 hour the antibody was removed from the membrane and 20 mL of antibody wash buffer (WesternBreeze kit) applied to the membrane and incubated with agitation for 5 minutes at room temperature. After 5 minutes the wash buffer was removed from the membrane. This washing step was repeated twice more. After washing, 10 mL of pre-diluted alkaline phosphatase conjugated anti-rabbit IgG secondary antibody (WesternBreeze kit) was applied onto the membrane and incubated at room temperature for 30 minutes. Membrane was washed with antibody wash as described above and then washed for two 5 minute washes in 20 mL of RO water. After the final wash, water was removed and 5 mL of BCIP/NBT substrate (WesternBreeze kit) added. The membrane was developed at room temperature until bands appeared (5-30 minutes) and reaction quenched by two 5 minute washes in 20 mL of RO water. The membrane was air dried then imaged on a ChemiGenius Bioimaging System.

Growth media

Minimum Essential Media, MEM

10% heat-inactivated fetal bovine serum, FBS

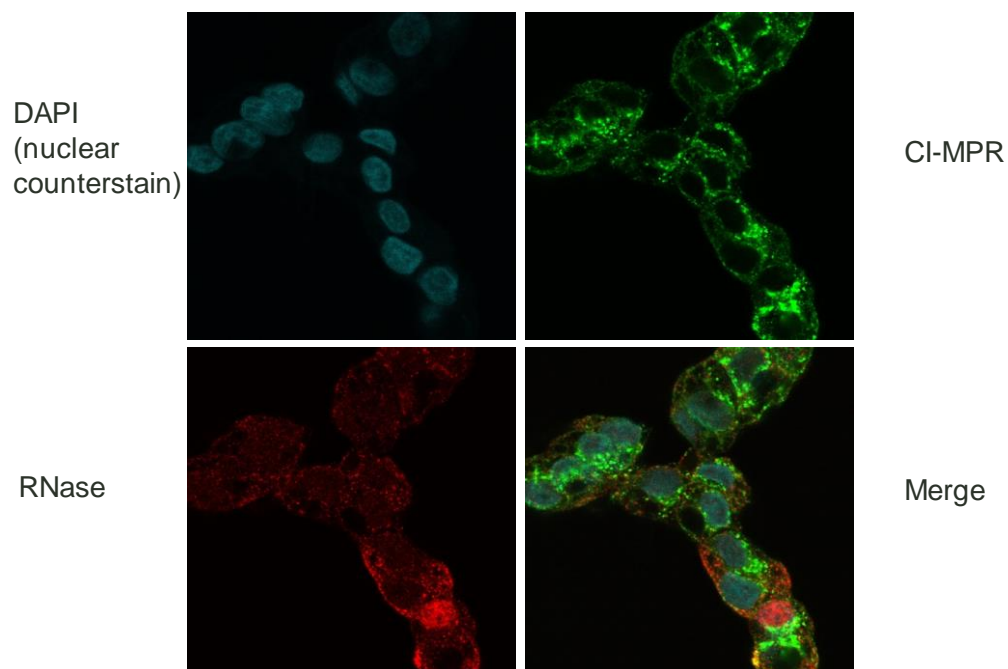
100U penicillin

100 µg/ml streptomycin

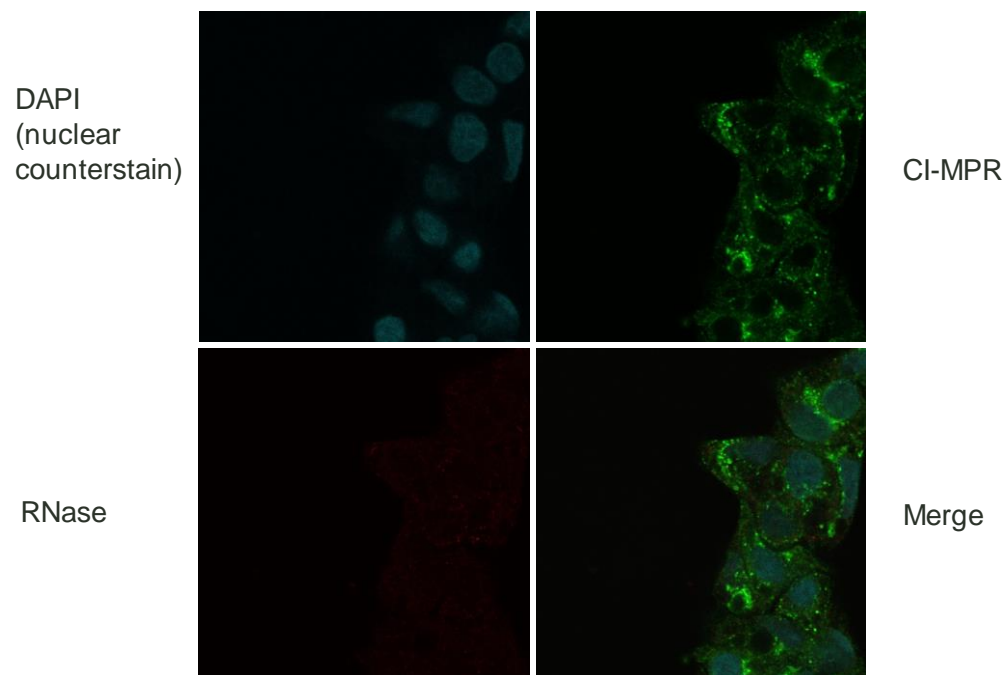
250 ng/ml amphotericin

Filter sterilized through a 0.22 µm filtration unit. stored at 4 °C.

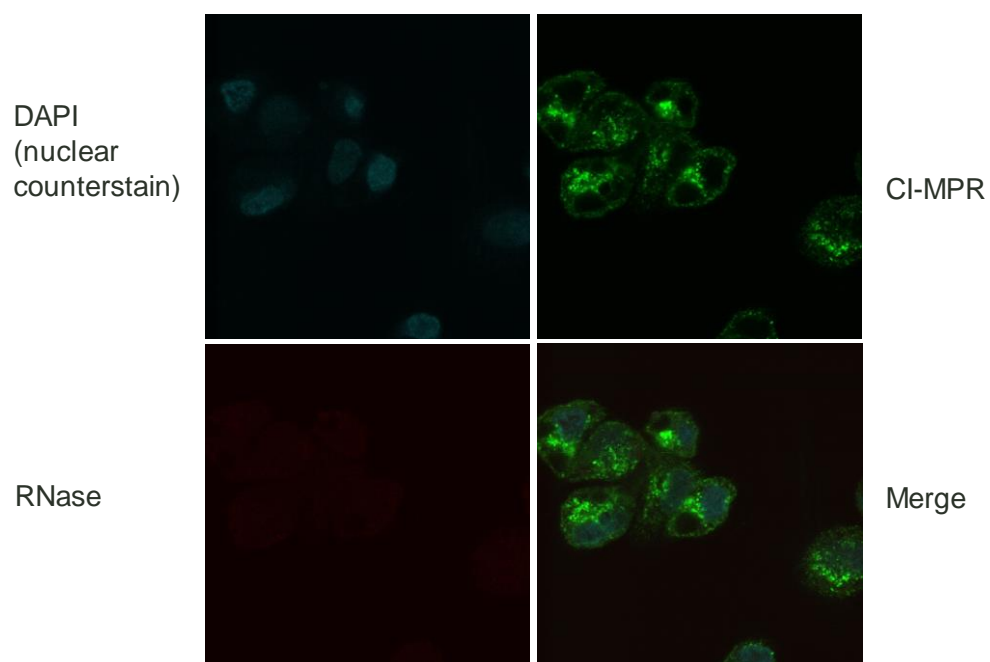
A.4 Co-localization of the CI-MPR and RNase (Imaging studies)



A.5 deglycosylated RNase B(Imaging studies)



A.6 No RNase (Imaging studies)



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